Ecotoxicology of metal-hydrocarbon mixtures in benthic invertebrates

Kurt A. Gust
Louisiana State University and Agricultural and Mechanical College, kgust1@lsu.edu

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ECOTOXICOLOGY OF METAL-HYDROCARBON MIXTURES IN BENTHIC INVERTEBRATES

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

The Department of Biological Sciences

by
Kurt A. Gust
B.S., Saginaw Valley State University, 1999
May 2005
ACKNOWLEDGEMENTS

I thank Mary Lou and Paul J. Gust for being the best parents a person could ever wish to have. They have been pillars of support to me my whole life. I thank them for raising me to believe that I can accomplish anything I apply my mind, body, soul and energy to. This dissertation is yet another example that they were right. I also give enormous thanks for the opportunity to be mentored by a true Zen Master of Science, Dr. John W. Fleeger. I can think of no other person that I would have trusted more to guide me through the doctoral process than him. I thank all of my committee members including Dr. Kevin Carman, Dr. Joseph Siebenaller, Dr. Ralph Portier and Dr. Huiming Bao for their critical review of my dissertation research and professional development. They, in concert with Dr. Fleeger were masters of applying the “carrot” and the “stick”. I thank Dr. Danny Reible and all at the Department of Chemical Engineering who have been active contributors to my research. I thank Dr. Reible, again in concert with Dr. Fleeger, for funding my research through the Hazardous Substances Research Center, South and Southwest. I also thank Sigma Xi Grants-in-Aid of Research and Minerals Management Services for research funding. Peter Bathum, Aaron Martin, Emily Brumfield, Jamie Maiaro, Catherine Sutera, and Philippe Pucheu all deserve extra special thanks for their assistance in conducting experiments. I thank Dr. William Wallace, Dr. Paul Klerks, Dr. Guglielmo Tita, Dr. Roderic Millward and Dr. Xiaoxia Lu for their intellectual contributions to my research. Last but not least, I would like to thank all of my friends (more correctly stated, all of my brothers and sisters) for being part of some of the best times I have ever had in my life during my time as a graduate student at Louisiana State University. I thank them for helping to keep me human during the rigors of my doctoral training.
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ABSTRACT

Metal-hydrocarbon mixtures are becoming increasingly prevalent in natural environments due to expanding industrial activity and urbanization. The ecotoxicology of metal-hydrocarbon mixtures in benthic environments is of particular concern because both classes of contaminants partition to sediments and can thereby exert toxic effects in benthic organisms. Mixtures of dissimilar chemicals (including metals and hydrocarbons) are broadly hypothesized to elicit independent toxic effects however; this hypothesis has little supporting data. The purpose of this dissertation was to test this hypothesis for metal-hydrocarbon mixtures using environmentally relevant exposures and to determine mechanisms for observed interactive effects. Sediment and water-only bioassays were conducted employing the toxic heavy metal cadmium (Cd) and the polynuclear aromatic hydrocarbon phenanthrene (Phen) as model toxicants. Lethal and sublethal effects of singular and combined contaminants were examined in two freshwater species, the epibenthic amphipod Hyalella azteca and the bulk deposit-feeding benthic oligochaete Ilyodrilus templetoni. When interactive toxicity was observed, mixture effects on contaminant bioavailability, bioaccumulation and elimination were tested. As well, mixture effects on bioenergetics parameters were investigated and kinetic modeling was conducted to establish the source of mixture-mediated changes in contaminant bioaccumulation in I. templetoni. Cadmium-Phen mixtures caused independent effects in water-only exposures, but when incorporated into sediments, elicited synergistic lethal effects in H. azteca and antagonistic lethal effects in I. templetoni. Interactive effects were likely caused by Phen-mediated alterations in Cd bioaccumulation that resulted from changes in exposure via feeding.

The current basis for assessing ecotoxicological effects of contaminant mixtures in natural environments relies heavily on models derived from dosage-based mixture toxicology
with considerably less emphasis on environmental science and biology. Understanding how contaminants interact toxicologically is important, but does not provide all the information necessary for assessing effects in natural populations that encounter contaminant mixtures in a diversity of natural environments. My experiments indicate that exposure source may be more important than dosage-based toxicological interactions in determining contaminant mixture effects in sediment environments. If this trend is widespread, understanding how species are exposed, determining the route of uptake and understanding how environmental characteristics affect exposure may be more important in determining mixture effects than mixture toxicology.
CHAPTER 1

GENERAL INTRODUCTION
Understanding the ecotoxicology of metal-hydrocarbon mixtures is of great concern because the co-occurrence of these contaminants in natural environments has increased as a result of expanding industrial activity and urbanization (Callender and Rice 2000, Van Metre et al. 2000, Sanger et al. 1999a, 1999b). The effects of metal-hydrocarbons mixtures in natural environments are poorly understood. Although contaminants with dissimilar modes of toxic action (including metal-hydrocarbon mixtures) are generally hypothesized to elicit independent joint toxic effects (Escher et al. 2002), the literature review provided in Chapter 2 and the results of new investigations presented in Chapters 3-6 of this dissertation indicate that this hypothesis may not be accurate for metal-PAH mixtures.

In order to understand potential environmental toxicity of contaminant mixtures, chemical-mixture toxicology must be considered. Types of joint toxicity include the following: concentration addition or “simple similar action” is a non-interactive process in which the toxicity of the chemical mixture is proportional to the sum toxicity of each individual contaminant concentration (Price et al. 2002, Cassee et al. 1998, Broderius 1991). Independence, also known as “response addition” occurs when the contaminants in a mixture have no influence on each other’s toxic effects resulting in less than concentration additive toxicity (Price et al. 2002, Cassee et al. 1998, Broderius 1991). Independent joint toxicity has been demonstrated for dissimilarly acting organic compounds including industrials contaminants (Broderius et al. 1995) and pharmaceutical compounds (Faust et al. 2000). Interactions, “synergism” or “antagonism” occur when contaminants interact to produce toxic effects that are either much greater or much less than expected compared to the effect of either contaminant alone (Cassee et al. 1998, Broderius 1991).

The toxicity of metals-hydrocarbon mixtures in aquatic environments is of particular concern, especially in the benthos where these contaminants tend to accumulate. Both metals
and hydrocarbons (particularly those with high hydrophobicity) tend to partition from the water column into benthic sediments (Di Toro and McGrath 2000, Ankley et al. 1994, Di Toro et al. 1991). Sediments frequently become a long-term repository for contaminants eliciting toxic effects in benthic organisms. The ecotoxicology of metal-hydrocarbon mixtures in benthic invertebrates was investigated in a series of experiments. A wide variety of experimental techniques were utilized not only to assess metal-hydrocarbon toxicity, but to elucidate mechanisms causing observed interactive effects.

The highly toxic heavy metal cadmium (Cd) and a representative of the most toxic class of non-substituted hydrocarbons, the poly-nuclear aromatic hydrocarbon (PAH) phenanthrene, were used as model contaminants for assessing joint-toxic effects of metal-hydrocarbon mixtures. Both contaminants are environmentally ubiquitous and are associated with industrial and urban contamination. Joint-toxic effects were investigated in two phylogenetically distinct species of freshwater invertebrates, each having unique associations with benthic sediments. The tubificid oligochaete *Ilyodrilus templetoni* (Southern) is a bulk deposit-feeding annelid that throughout its life history is predominantly found burrowed in sediments. The freshwater amphipod and U.S. Environmental Protection Agency standard test species *Hyalella azteca* (Saussure) is a selective deposit feeder that may burrow superficially in sediments or perch on benthic detritus. The unique life histories, differences in associations with sediments, and distant phylogenies of these species provide an opportunity to test the importance of biology on contaminant mixture toxicology.

In Chapter 3, the joint toxicity of Cd and phenanthrene (Phen) was investigated in *H. azteca* in sediment and water-only exposures. Ten-day U.S. Environmental Protection Agency standard test methods for evaluating sediment toxicity (USEPA, 2000) were used to assess Cd-
Phen joint toxicity. In sediments exposures, the effects of individual and combined contaminants were investigated for both lethal and growth rate endpoints. Effects of Cd-Phen mixtures on lethality were also tested in aqueous exposures and compared with mixture effects observed in sediments. This study is in press in *Archives of Environmental Contamination and Toxicology*.

In Chapter 4, the effect of Phen on Cd bioaccumulation in sediment and water-only exposures was tested in *H. azteca*. Additionally, the affects of Phen on water quality parameters associated with Cd bioavailability and effects of Phen on dissolved Cd concentrations in overlying water were tested. These investigations were conducted to elucidate the causes of interactive joint toxicity observed in Chapter 2. This study is in revision after submission for publication in *Environmental Toxicology and Chemistry*.

In Chapter 5, the effects of Cd, Phen and the Cd-Phen mixture were investigated in *I. templetoni*. Effects of contaminants were investigated in sediment exposures for endpoints including lethality, tissue necrosis, feeding rate, and burrowing avoidance. The interconnection between observed Cd-Phen mixture effects on sublethal endpoints and the interactive effect of the mixture on lethality are also discussed.

In Chapter 6, a series of experiments were conducted in an attempt to elucidate the causes of interactive lethal effects observed in *I. templetoni* exposed to Cd-Phen mixtures. Effects of Phen on Cd bioaccumulation and elimination were tested in both sediment and water-only exposures. Additionally, the effects of Cd on Phen bioaccumulation and biota sediment accumulation factor (BSAF) were tested. Effects of Phen on Cd bioavailability in sediments were tested using simultaneously extractable metal - acid volatile sulfide (SEM - AVS) relationships and tests of Phen effects on Cd concentrations in porewater and overlying water. Finally, a bioenergetic kinetic model utilizing empirically derived model parameter values was
used to elucidate the mechanisms responsible for interactive toxic effects observed between Cd and Phen in *I. templetoni*.

A final conclusions section summarizes the studies presented in this dissertation. The importance of metal-hydrocarbon mixture effects will be discussed in light of the novel observations uncovered in this body of work and the literature review presented in Chapter 2.

**LITERATURE CITED**


Escher BI, Hermens JLM (2002) Modes of action in ecotoxicology: Their role in body burdens, species sensitivity, QSARs, and mixture effects. Environ Sci and Technol 36:4201-4217


CHAPTER 2

REVIEW OF METAL-PAH JOINT TOXICITY STUDIES
INTRODUCTION

The complexity of joint-toxic responses to heavy metals and PAHs makes environmental risk assessment difficult, and may reduce the predictive power of existing assessment protocols. Currently, the most conservative model for environmental hazard assessment assumes concentration-additive toxicity between chemicals in a mixture (USEPA 2000). Less conservative joint-toxicity models have been proposed for use in hazard assessment in which independent joint toxicity between dissimilar chemicals (Altenburger et al. in press, Price et al. 2002, Faust et al. 2000). Based on the comprehensive review of metal-PAH joint toxicity literature presented herein, hypotheses of independent and/or concentration-additive joint toxicity are poor predictors of toxic effects elicited by combined metals and PAHs. Furthermore, a high proportion of the studies (22 of 31 papers) indicated that the occurrence of synergistic toxicity between these chemical classes may be predominant (Tables 2.1 and 2.2). These observations provide probable cause for regulators to reconsider the underlying assumptions used for risk assessment of metal-PAH mixtures. Further, improved understanding of metal-hydrocarbon joint toxicity from environmental sources must also be considered. The following literature review summarizes metal-PAH effects that have been observed to range from pharmacological interactions through ecosystem level responses.

Overview and Approach

Although relatively few studies have examined joint metal-PAH toxicity (see below), results to date are complex including examples of additive, independent and interactive (antagonistic and synergistic) toxic effects (Tables 2.1 and 2.2). Additionally, the literature suggests that these responses vary among species, among toxicological endpoints within species and may also vary within endpoints. Before reviewing the literature, it is important to address the
criteria used to distinguish toxic interactions from concentration additive or independent responses. A strict definition of a toxicological interaction implies that the absorption, distribution, biotransformation, excretion or toxicodynamics of one contaminant is influenced by another (Cassee et al. 1998, Broderius 1991). For the purposes of this review, this definition will be expanded to include observed interactive effects on individuals (including mortality and sublethal endpoints) and at higher levels of ecological organization.

Literature review of metal-PAH mixture investigations (focusing primarily on aquatic organisms) was collected over a 5-year period using a variety of search techniques including web-based searches on the Web of Science and PubMed.com. The majority of the literature (26 of 31 papers) accumulated for this review provided at least one example of interactive toxicity or mechanisms by which interactive toxicity may occur between the metals and PAHs tested. The overwhelming representation of metal-PAH interactions is undoubtedly biased by selective submission and/or acceptance of papers finding interactive effects. Regardless, the literature suggests that joint-toxic interactions between metals and hydrocarbons are possible if not probable. These interactive effects must be addressed when considering the toxicity of metal-PAH mixtures. The review will focus on the occurrences of interactive toxicity and attempt to establish possible mechanisms whereby interactions may occur.

DISCUSSION

Metal-PAH Joint Toxicity Literature

Van den Hurk et al. (1998a) found dose-dependent synergistic and antagonistic lethal joint-toxicity with combined doses of Cd and benzo[a]pyrene (BaP) in mummichogs (**Fundulus heteroclitus**). Sublethal doses of Cd combined with lethal BaP doses reduced mortality, whereas combined sublethal doses of Cd and BaP caused higher than expected mortality. Cadmium was
<table>
<thead>
<tr>
<th>Test Organism</th>
<th>Metal</th>
<th>Hydrocarbon</th>
<th>Endpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>grass shrimp (<em>Palaemonetes pugio</em>)</td>
<td>Ba, Ni, Zn</td>
<td>dibenzothiophene, napthalene, phenanthrene</td>
<td>contaminant acclimation</td>
</tr>
<tr>
<td>tubificid oligochaete (<em>Ilyodrilus templetoni</em>)</td>
<td>Cd</td>
<td>phenanthrene</td>
<td>lethality</td>
</tr>
<tr>
<td>tubificid oligochaete (<em>Ilyodrilus templetoni</em>)</td>
<td>Cd</td>
<td>phenanthrene</td>
<td>feeding rate</td>
</tr>
<tr>
<td>freshwater amphipod (<em>Hyalella azteca</em>)</td>
<td>Cd</td>
<td>phenanthrene</td>
<td>lethality</td>
</tr>
<tr>
<td>microcosms of a natural salt marsh community</td>
<td>Cu, Cd, Hg, Cr, Pb</td>
<td>diesel fuel</td>
<td>abundances of native benthic invertebrates</td>
</tr>
<tr>
<td>freshwater amphipod (<em>Hyalella azteca</em>)</td>
<td>Methyl-Hg</td>
<td>Dieldrin (pesticide)</td>
<td>lethality</td>
</tr>
<tr>
<td>freshwater amphipod (<em>Hyalella azteca</em>)</td>
<td>Methyl-Hg</td>
<td>Chlorpyrifos (pesticide)</td>
<td>lethality</td>
</tr>
<tr>
<td>mummichog (<em>Fundulus heteroclitus</em>)</td>
<td>Cd</td>
<td>benzo[a]pyrene</td>
<td>lethality</td>
</tr>
<tr>
<td>European sea bass (<em>Dientrarchus labrax</em>)</td>
<td>Cd</td>
<td>benzo[a]pyrene</td>
<td>phagocytic index and activity in kidney and spleen</td>
</tr>
<tr>
<td>sheepshead minnow (<em>Cyprinodon variegatus</em>)</td>
<td>Zn</td>
<td>phenanthrene</td>
<td>lethality</td>
</tr>
<tr>
<td>sheepshead minnow (<em>Cyprinodon variegatus</em>)</td>
<td>Ba, Ni, Zn</td>
<td>dibenzothiophene, napthalene, phenanthrene</td>
<td>inheritance of resistance</td>
</tr>
<tr>
<td>English sole (<em>Parophrys vetulus</em>)</td>
<td>Cd</td>
<td>Aroclor 1254 (PCB)</td>
<td>decreased occurrence of hepatic &amp; intestinal lesions</td>
</tr>
<tr>
<td>freshwater fish (<em>Coregonus sp.</em>)</td>
<td>As</td>
<td>Aroclor 1254 (PCB)</td>
<td>lethality</td>
</tr>
<tr>
<td>duckweed (<em>Lemna gibba</em>)</td>
<td>Cu</td>
<td>oxygenated PAH</td>
<td>photosynthetic rate</td>
</tr>
<tr>
<td>Microtox -- On Contaminated Bayou Samples</td>
<td>complex mixture</td>
<td>PAHs and other hydrocarbons</td>
<td>Microtox EC50</td>
</tr>
<tr>
<td>Joint-Toxicity</td>
<td>Authors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>---------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. synergistic</td>
<td>Klerks 1999</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. antagonistic</td>
<td>Gust and Fleeger submitted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. independent</td>
<td>Gust and Fleeger submitted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. synergistic</td>
<td>Gust in press</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. &quot;synergism&quot;, dose addition and independence</td>
<td>Millward et al 2004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. independent</td>
<td>Steevens and Benson 2001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. dose-additive</td>
<td>Steevens and Benson 1999, 2001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. synergistic and antagonistic</td>
<td>van den Hurk et al. 1998a, 1998b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. synergistic</td>
<td>Lemaire-Gony et al. 1995</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. synergistic and antagonistic</td>
<td>Moreau et al. 1999</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. synergistic</td>
<td>Klerks and Moreau 2001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12. antagonistic</td>
<td>Rhodes et al. 1985</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13. possible synergism</td>
<td>Passino and Kramer 1980</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14. synergistic</td>
<td>Babu et al. 2001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15. synergistic and antagonistic</td>
<td>Mowat and Bundy 2002</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Numbers (1-15) correspond with observations listed in Table 2.1.
found to impair BaP metabolism in mummichogs by inhibiting hepatic cytochrome P450 (Cyp1A) enzyme activity (van den Hurk et al. 1998b). Inhibition of cytochrome P450 enzyme production and lowering of overall hydrocarbon metabolism, as measured by 7-ethoxyresorufin-\(O\)-deethylase (EROD) activity, by heavy metals has been found in a variety of fish species (Viarengo et al. 1997, Brüschweiler et al. 1996, Viarengo et al. 1987, Fair 1986, Förlin et al. 1986, George 1989, George and Young, 1986) and was initially characterized in rodents (Eaton et al. 1980, Schnell et al. 1979, Yoshida et al. 1976, Unger and Clausen 1973). Although this appears to be a general trend, evidence of increased EROD activity resulting from combined metal-PAH exposure also exists (Lemaire-Gony et al. 1995, Lemaire-Gony and Lemaire 1992). 

Cytochrome P450 pathways allow organisms to biotransform, detoxify and excrete an array of organic xenobiotics (Casarett and Doull 2001). However, this process may create intermediate metabolites that are more toxic than the parent compound. Such is the case with PAH degradation. Specifically, aryl hydrocarbon hydroxylase (AHH) activity initiates PAH metabolism creating polar intermediates and highly-reactive toxic metabolites (Casarett and Doull 2001). GSH-S-transferase activity catalyzes reactions reducing the toxicity of these reactive metabolites and facilitates their excretion (Fair 1986). It is suggested that the balance between these reactions is crucial in determining hydrocarbon toxicity, and metals have been shown to elicit differential effects depending on the rate of each reaction (Fair 1986, Lemaire-Gony and Lemaire 1992). Therefore, metals may facilitate either synergistic or antagonistic toxic effects depending on which reactions they affect during PAH metabolism.

In addition to metals altering the toxicity of hydrocarbons, interactive toxicity may also be elicited when hydrocarbons influence metal toxicity. George and Young (1986) showed that the PAH 3-methylcholanthrene delayed the onset of metallothionein induction in Plaice,
Table 2.2. Review of metal-PAH joint toxicity studies emphasizing biochemical and molecular toxicology. The article highlighted in gray involves joint toxicity of a non-PAH hydrocarbon and a metal.

<table>
<thead>
<tr>
<th>Test Organism</th>
<th>Metal</th>
<th>Hydrocarbon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. marine mussel (<em>Mytilus edulis</em>)</td>
<td>Cu</td>
<td>phenanthrene</td>
</tr>
<tr>
<td>2. marine mussel (<em>Mytilus galloprovincialis</em>)</td>
<td>Cd</td>
<td>phenanthrene</td>
</tr>
<tr>
<td>3. European sea bass (<em>Dientrarchus labrax</em>)</td>
<td>Cd</td>
<td>benzo[a]pyrene</td>
</tr>
<tr>
<td>4. European sea bass (<em>Dientrarchus labrax</em>)</td>
<td>Cd</td>
<td>benzo[a]pyrene</td>
</tr>
<tr>
<td>5. European sea bass (<em>Dientrarchus labrax</em>)</td>
<td>Cu, Hg, Methyl-Hg</td>
<td>BaP, β-naphthoflavone</td>
</tr>
<tr>
<td>6. European eel (<em>Anguilla anguilla</em>)</td>
<td>Cd</td>
<td>benzo[a]pyrene</td>
</tr>
<tr>
<td>7. black sea bass (<em>Centropristis striata</em>)</td>
<td>Cd</td>
<td>benzo[a]pyrene</td>
</tr>
<tr>
<td>8. black sea bass (<em>Centropristis striata</em>)</td>
<td>Cd</td>
<td>naphthalene</td>
</tr>
<tr>
<td>9. plaice (<em>Pleuronectes platessa</em>)</td>
<td>Cd</td>
<td>3-methylcholanthrene (model PAH)</td>
</tr>
<tr>
<td>10. plaice (<em>Pleuronectes platessa</em>)</td>
<td>Cd</td>
<td>3-methylcholanthrene (model PAH)</td>
</tr>
<tr>
<td>11. rainbow trout (<em>Salmo gairdneri</em>)</td>
<td>Cd</td>
<td>organic xenobiotics (general)</td>
</tr>
<tr>
<td>12. fish hepatoma cell culture PLHC-1</td>
<td>Cd, Co, Cu, Ni, Pb, Zn</td>
<td>3-methylcholanthrene (model PAH)</td>
</tr>
<tr>
<td>Test Organism</td>
<td>Metal</td>
<td>Hydrocarbon</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>13. rat</td>
<td>Cd, Se, Mn, Pb, Ag, Cr</td>
<td>organic xenobiotics (general)</td>
</tr>
<tr>
<td>14. rat</td>
<td>Cd</td>
<td>organic xenobiotics (general)</td>
</tr>
<tr>
<td>15. mouse</td>
<td>Cd</td>
<td>organic xenobiotics (general)</td>
</tr>
<tr>
<td>16. mouse</td>
<td>Cd</td>
<td>hexobarbital</td>
</tr>
</tbody>
</table>
Table 2.2. (Continued)

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Joint-Toxicity</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>increased oxygen consumption and ammonium excretion</td>
<td>&quot;synergistic&quot;</td>
<td>Moore et al. 1984</td>
</tr>
<tr>
<td>no affect of phenanthrene on Cd bioaccumulation and metallothionein binding. No difference in lysosome stability</td>
<td>&quot;independent&quot;</td>
<td>Viarengo et al. 1987</td>
</tr>
<tr>
<td>independent effect on Na/K-ATPase activity</td>
<td>&quot;independent&quot;</td>
<td>Lemaire-Gony et al. 1995</td>
</tr>
<tr>
<td>combined Cd and BaP cause enhanced EROD activity</td>
<td>&quot;antagonistic&quot;</td>
<td>Lemaire-Gony et al. 1995</td>
</tr>
<tr>
<td>EROD activity in hepatic microsomes inhibited by metals</td>
<td>&quot;synergistic&quot;</td>
<td>Viarengo et al. 1997</td>
</tr>
<tr>
<td>combined Cd and BaP cause enhanced EROD, BaP hydroxylase and GST activity</td>
<td>&quot;antagonistic&quot;</td>
<td>Lemaire-Gony and Lemaire 1992</td>
</tr>
<tr>
<td>inhibition of GSH-S-Transferase and BaP hydroxylase activity caused by Cd</td>
<td>&quot;synergistic&quot;</td>
<td>Fair 1986</td>
</tr>
<tr>
<td>increased metal assimilation from food (oyster tissue) in presence of naphthalene</td>
<td>&quot;synergistic&quot;</td>
<td>Fair and Sick 1983</td>
</tr>
<tr>
<td>delayed metallothionein induction</td>
<td>&quot;synergistic&quot;</td>
<td>George and Young 1986</td>
</tr>
<tr>
<td>inhibition of EROD activity, Reduced GSH transferase, GSH peroxidase and glucuronyl transferase activity</td>
<td>&quot;synergistic&quot;</td>
<td>George and Young 1986</td>
</tr>
<tr>
<td>inhibition of phase I and II organic xenobiotic biotransformation activities in liver and kidney</td>
<td>&quot;synergistic&quot;</td>
<td>Förlin et al. 1986</td>
</tr>
<tr>
<td>inhibition of Cytochrome P450 (CYP1A) and reduced EROD activity</td>
<td>&quot;synergistic&quot;</td>
<td>Brüschweiler et al. 1996</td>
</tr>
</tbody>
</table>

Numbers (1-12) correspond with observations listed in Table 2.2.
Table 2.2. (Continued)

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Joint-Toxicity</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>15. Cytochrome P450 inhibition</td>
<td>&quot;synergistic&quot;</td>
<td>Unger and Clausen 1973</td>
</tr>
</tbody>
</table>

Numbers (13-16) correspond with observations listed in Table 2.2.
Pleuronectes platessa hepatocytes by 6 days compared to organisms exposed to Cd alone.

Metallothioneins are evolutionarily wide-spread, low molecular weight proteins that bind to various metals in situ significantly reducing the toxicity of those metals (Roesijadi 1992). Thus, inhibition of metallothionein induction by PAHs may cause relatively modest metal body burdens to elicit toxicity. Although this is a probable mechanism of synergistic toxicity, it is difficult to establish this mechanism as a general trend. For example, Viarengo et al. (1987) demonstrated that phenanthrene had no effect on Cd-metallothionein binding or total metal body burden in the marine mussel Mytilus galloprovincialis when exposed to the contaminants in aqueous solution.

PAHs have been found to affect the bioaccumulation of metals. Fair and Sick (1983) found that the Black Sea Bass, Centropristis striata assimilated higher concentrations of Cd in various tissues from food (spiked oyster tissue) containing Cd and naphthalene compared to food containing Cd alone. Conversely, phenanthrene reduced Zn bioaccumulation from sediments in sheephead minnow Cyprinodon variegatus, and this reduction was associated with antagonistic lethal toxicity (Moreau et al., 1999). Similarly, phenanthrene was found to decrease the bioaccumulation of Cd from sediments in the tubificid oligochaete Ilyodrilus templetoni (Chapter 6) also eliciting antagonistic lethal toxicity (Chapter 5). In the studies described above, metals did not significantly alter the bioaccumulation of PAHs.

Examples of interactive toxicity between metals and PAHs are not exclusive to animals. Babu et al. (2001) found synergistic inhibitory effects on photosynthetic rate and plant growth in duckweed (Lemna gibba), when exposed to an oxygenated PAH (1,2-dihydroxyanthraquinone) and Cu. The combined contaminants were found to dismantle photosynthetic electron-transport chain function thereby severely inhibiting energy metabolism and growth. Neither contaminant elicited this effect alone.
Contaminant interactions that alter sublethal and/or subcellular toxicity may affect species’ ability to survive in natural environments. Lemaire-Gony et al. (1995) found synergistic immuno-toxicological affects caused by combined Cd and BaP exposure in the European sea bass *Dentarchus labrax*. The combined contaminants significantly reduced phagocytic index and phagocytic activity thereby increasing the fishes’ susceptibility to infectious diseases. The combination of phenanthrene and Cu caused synergistic increases in oxygen consumption and ammonia production in the marine mussel *Mytilus edulis* (Moore et al. 1984). Increased oxygen demand in sessile organisms such as *M. edulis* could prove to be deleterious during periods of hypoxia. Klerks (1999) found that grass shrimp’s (*Palaemonetes pugio*) physiological acclimation to metal or PAH stress was reduced when pre-exposed to mixtures of metals and PAH. Additionally, Klerks and Moreau (2001) showed that the heritability of resistance to metal or PAH stress in *Cyprinodon variegatus* decreased as the number of components in the metal-PAH mixture increased. The evolutionary implications of population restructuring resulting from exposure to contaminant mixtures are poorly understood.

Assessing the effects of combined metal-PAH contamination in ecosystems is additionally complicated by dynamics not considered in biochemical or population bioassay experiments. In addition to the direct toxic action of contaminants on tissues, individuals and populations, ecosystem-level responses include possible contaminant-induced indirect ecological effects (Fleeger et al. 2003) and alteration of environment-specific biogeochemistry which mediates contaminant flux and hence organismal exposure. Millward et al. (2004) demonstrated apparent synergistic effects of combined metals and diesel fuel on the copepod abundance in salt marsh microcosms. The apparent synergism was suggested to be the result of a dampened indirect effect where “diesel-resistant” copepods that proliferated by population-level responses
in diesel-only exposures were inhibited by metals. The microcosm study also suggested that diesel enhanced Cu and Cr retention in sediment while metals enhanced the efflux of total petroleum hydrocarbons (Millward et al. 2004). The differential retention/efflux of contaminants caused by co-contaminants may alter exposure to and hence the overall toxicity to organisms in natural environments.

Predictive models that have been developed to assess environmental quality have dealt with contaminant mixtures in a number of ways. Mowat and Bundy (2002) detected interactive (synergistic and antagonistic) toxic effects in Microtox bioassays at all 10 sites examined in two Louisiana bayous containing complex metal-hydrocarbon mixtures. In the above study, the ability to accurately assess environmental impact may have been confounded by combined metal-PAH contamination.

Although not reviewed here, interactive toxicity has also been shown to occur between heavy metals and organic contaminants other than PAHs (Steevens et al. 2001, 1999, Rhodes et al. 1985, Passino and Matsumoto 1980). Although, the interactive metal-hydrocarbon effects observed in these studies indicate that further investigation of metals combined with non-PAH hydrocarbons may also deviate from the hypothesis of independent joint toxicity.

**Summary and Conclusions**

In summary, the majority of studies investigating metal-PAH joint toxicity suggest contaminant interactions occur frequently, and arise through a variety of mechanisms. The hypothesis that chemicals with dissimilar modes of toxic action elicit independent joint-toxic effects was poorly supported. Furthermore, the majority of the studies reviewed suggested the joint toxicity of metals and PAHs is synergistic. These observations indicate that the assumption of independent joint toxicity between metals and PAHs is not correct, and should not be used in
assessment of environmental quality. An improved understanding of the interrelationships among pharmacological, organismal, population-level and ecosystem-level effects are required to achieve a holistic understanding of metal-PAH effects in natural environments.

LITERATURE CITED


Fair PH (1986) Interaction of benzo(a)pyrene and cadmium on GSH-S-transferase and benzo(a)pyrene hydroxylase in the black sea bass Centropristis striata. Arch Environ Contam Toxicol 15:257-263


Gust KA (in press) Joint toxicity of cadmium and phenanthrene in the freshwater amphipod *Hyalella azteca*. Arch Environ Contam Toxicol In press:

Klerks PL (1999) Acclimation to contaminants by the grass shrimp *Palaemonetes pugio*: individual contaminants vs. mixtures. Ecotoxicology 8:277-286

Klerks PL, Moreau CJ (2001) Heritability of resistance to individual contaminants and to contaminant mixtures in the sheepshead minnow (*Cyprinodon variegatus*). Environ Toxicol Chem 20:1746-1751


Moreau CJ, Klerks PL, Haas CN (1999) Interaction between phenanthrene and zinc in their toxicity to the sheepshead minnow (*Cyprinodon variegatus*). Arch Environ Contam Toxicol 37:251-257


Roesijadi G (1994) Metallothionein induction as a measure of response to metal exposure in aquatic animals. Environ Health Perspectives 102:91-96


US Environmental Protection Agency (2000) Supplementary guidance for conducting health risk assessment of chemical mixtures. EPA Office of Research and Development EPA/630/R-00/002


Viarengo A, Bettella E, Fabbri R, Burlando B, Lafaurie M (1997) Heavy metal inhibition of EROD activation in liver microsomes from the bass Dicentrarchus labrax exposed to organic xenobiotics: Role of GSH in the reduction of heavy metal effects. Mar Environ Res 44:1-11


CHAPTER 3

JOINT TOXICITY OF CADMIUM AND PHENANTHRENE IN THE FRESHWATER AMPHIPOD HYALELLA AZTECA*

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INTRODUCTION

Mixtures of heavy metals and poly-nuclear aromatic hydrocarbons (PAH) are becoming increasingly prevalent in benthic and wetland sediments as a result of urbanization and industrial contamination (Sanger et al. 1999a, 1999b). The joint-toxicity of these contaminants to organisms in natural environments can be complex and is related to the chemistries of the individual compounds, environment-specific bioavailability, toxicological modes of action and possible pharmacological interactions among contaminants once bioaccumulated (Cassee et al. 1998, Broderius, 1991). Current hypotheses regarding chemicals with “dissimilar” toxicology (i.e., metals and hydrocarbons) suggest their joint toxicity is independent (Price et al. 2002, Broderius 1991). However, published studies that have investigated the joint-toxicity of metals and PAHs indicate that toxicity may be concentration-additive, independent, synergistic or antagonistic (Millward et al. 2004, van den Hurk et al. 1998b, Babu et al. 2001, Moreau et al. 1999, Gust and Fleeger submitted). These responses have also been observed to vary among species (Lemaire-Gony and Lemaire 1992, George and Young 1986), among toxicological endpoints (Lemaire-Gony et. al. 1995, Gust and Fleeger submitted) and within endpoints (Moreau et al. 1999, van den Hurk et al. 1998a).

The purpose of the present study was to examine the joint toxicity of metal-PAH mixtures in *Hyalella azteca* using cadmium (Cd) and phenanthrene (Phen) as model contaminants in both sediment- and aqueous-toxicity bioassays. *Hyalella azteca* is a toxicant-sensitive epibenthic freshwater amphipod that is routinely used in standard testing procedures designed to assess contaminant toxicity and sediment quality (USEPA 2000, ASTM 2003). The efficacy of these bioassays for assessing impact in combined metal-PAH exposures was investigated. It has been suggested that metals bioaccumulate and exert toxicity in *H. azteca*
primarily through exposure to the overlying water associated with contaminated sediment (Borgmann et al. 2001, Borgmann and Norwood 1999, Warren et al. 1998). As well, equilibrium partitioning theory indicates that the dissolved fraction of nonionic organic chemicals (i.e., PAH) in sediment porewater is responsible for contaminant bioaccumulation and toxicity in aquatic organisms (Di Toro et al. 1991). Although the contribution of dissolved metal and PAH to toxicity are each individually well demonstrated, studies including Ingersoll et al. (2000) and Wang and Fisher (1999) indicate that benthic invertebrates’ exposure to sediment-associated contaminants can influence bioaccumulation and toxicity. Furthermore, the combined effects of these contaminants in sediment exposures are unknown, and it is unclear if the combined effects are equivalent in sediment and aqueous exposures. We hypothesized that Cd and Phen would have independent joint-toxic effects in *H. azteca* and that there would be no difference in *H. azteca*’s response to combined Cd and Phen when comparing sediment and aqueous exposures. Potential causative mechanisms explaining deviations from the above hypotheses are discussed and posed for future investigation.

**MATERIALS AND METHODS**

**Laboratory Culture and Test Organisms**

*Hyalella azteca* (Saussure) cultures were initiated in fall of 2002, and were maintained in 20-L aquaria maintained at 23 ± 1°C. Aquaria were filled with dechlorinated tap water of which >50% volume was siphoned off and replaced 3 times wk⁻¹. Aquaria were individually aerated and experienced a 12L:12D photoperiod of indirect light. Amphipods were fed dried, senesced maple leaves that were added as needed.

**Test Methods**

All containers and apparatus used to conduct both sediment and aqueous experiments were acid cleaned prior to use. Sediment toxicity bioassays for Cd and Phen were conducted
based on U.S. Environmental Protection Agency method 100.1 for *Hyalella azteca* 10-d survival and growth (USEPA 2000). Ten randomly selected *H. azteca* were exposed in 400 mL plastic beakers containing 100 mL test sediment and 200 mL overlying dechlorinated tap water. Static–renewal, water-replacement procedures were conducted in which the entire water volume was replaced daily. All beakers were maintained at 23 ± 1°C with a 16L:8D photoperiod. The amphipods were acclimated to this photoperiod for 1 d prior to experiment initiation. Each sediment treatment consisted of 5 replicates, and treatment replicates were arranged and maintained in a completely randomized design. Sub-adult *H. azteca* (2-3 wk old) were collected by sieving through 1000- and 500-µm mesh, stacked sieves, and individuals retained on the 500-µm sieve were used in the toxicity bioassays (Driscoll et al. 1997b). This age class is recognized to be as sensitive to Cd as 1-2 wk old animals and their larger size increases ease of recovery and improves the accuracy of mortality determinations (USEPA 2000). During bioassays, animals were fed 1.5 mL aliquots of yeast, Cerophyl® and trout chow (YCT; USEPA 2000) daily. Observation of exposure chambers during bioassays suggested daily meals were ingested completely.

Dissolved oxygen concentration and temperature in overlying water were monitored daily in 3 randomly selected replicates using an Orion model 820 oxygen meter. Overlying-water pH and ammonia concentrations were measured from 5 randomly selected replicates at experiment initiation and termination. pH was measured with a Fisher Accumet® Model 805MP pH meter and ammonia concentrations were monitored using a NH₃/NH₄ aquarium test kit, Aquarium Pharmaceuticals, Inc. Chalfont, PA.

At experiment end, amphipods were collected by washing the contents of each beaker on a 250-µm sieve. Sieve contents were rinsed into a counting dish and the number of living and
dead amphipods was determined. Missing amphipods were considered to be dead and percent
mortality was calculated. Growth rate was quantified by subtracting the mean dry weight of 5
groups of 10 randomly selected individuals collected at experiment initiation from the dry weight
standardized by the number of surviving animals for each replicate at the end of the 10-d
bioassay.

Aqueous lethality bioassays for Cd and Phen were conducted using ten randomly selected
H. azteca inserted into 100 mL glass beakers containing 80 mL of treatment solution in 4
replicates. Glass beakers were used to minimize Phen binding to exposure chambers. Cadmium
and Phen were dissolved in dechlorinated-tap water to create treatment solutions. Phenanthrene
was first dissolved in a small volume of acetone carrier and then added to water. The acetone
concentration in water was 0.1 ml L\(^{-1}\) and a procedural control was conducted to test for acetone
effects. Static renewal-water replacement was conducted by replacing the entire water volume
twice daily. Test solutions were prepared no more than 1h prior to water renewal. Cadmium
concentrations in water-only exposures have been demonstrated to remain stable between static
renewals, whereas Phen concentrations may vary by nearly 50% (Gust, submitted). Therefore,
only initial Cd concentrations were measured and aqueous Phen concentrations were measured
before and after each renewal. Water quality parameters were measured as above.

**Sediment Preparation**

Sediment was collected from Bayou Manchac, a rural freshwater bayou near Baton
Rouge, Louisiana, USA that has had no history of industrial activity. Levels of trace and heavy
metals (Gust, unpublished) and PAHs (Lu, personal communication) detected in Bayou Manchac
sediment suggest only background concentrations. Sediment was sieved through a 1-cm screen
and frozen and thawed twice to eliminate native macroinvertebrates. Sediment was
homogenized and stored in the dark at 4°C. Total organic carbon (TOC) content of the sediment was determined to be 2.59% ± 0.23% using a Perkin Elmer 2400 CHN Series II elemental analyzer (Norwalk, CT, USA). Samples were refluxed for 6h in concentrated HCl to eliminate inorganic carbonate and then oven dried at 70°C prior to analysis. The wet:dry ratio of the saturated sediment was 2.2:1.

Phenanthrene (98% purity, Aldrich Chemical Co. Milwaukee, WI, USA) was amended to sediment by dissolving the chemical in HPLC-grade hexane and then volatilizing the solvent in a high-purity nitrogen gas stream to coat the inside walls of opaque glass jars. The appropriate mass of wet sediment to achieve a targeted concentration was calculated using the sediment wet:dry ratio, then that mass was added to each jar and tumbled on a roller mill at room temperature (23 ± 1°C) for 28 d. Phenanthrene concentrations were measured using high-performance liquid chromatography (HPLC). Two replicates of each Phen-amended sediment treatment were frozen, freeze-dried, homogenized, and pre-weighed quantities were transferred to a glass extraction vessel. Sixty mL of a 1:1 mixture of HPLC grade acetone and hexane was added to the dried sediments. The solvent-sediment combination was sonicated for 20 min and then allowed 24 h for solvent extraction of Phen. After extraction, the solution was reduced in volume by 90% via volatilization in high-purity nitrogen gas stream, and then brought up to the initial volume with acetonitrile. Samples were analyzed using a Hewlett-Packard 1100 series (Hewlett-Packard, Palo Alto, CA, USA) HPLC. Both aqueous Phen and sediment-Phen extract concentrations were determined by reverse phase-HPLC employing an HC-ODS Sil-X, 5 μm particle diameter packed column. Phen was monitored by UV detection. Five minutes of isocratic elution with acetonitrile / water (4:6) (v/v) were followed with linear gradient elution to 100% acetonitrile over 25 min at a flow rate of 0.5 mL / min (US EPA 1986)
Two-way ANOVAs comparing measured sediment-Phen concentrations at d-0 and d-10 suggested Phen concentrations were equivalent (p > 0.05).

Cadmium chloride (98% purity, Sigma Chemical Co. Saint Louis, MO, USA) was dissolved in deionized water, and then slowly amended to a specific mass of wet sediment (determined by wet:dry ratio of sediment) to achieve targeted sediment concentrations. Each Cd solution was added dropwise to sediment in plastic jars undergoing mixing with a handheld kitchen mixer. For joint-toxicity bioassays, Phen was amended to sediment (as above) prior to addition of Cd. Day 0 sediment Cd concentrations were measured using inductively-coupled argon plasma spectrophotometry (ICP-AES). Two replicates of each Cd treatment were freeze-dried, milled and weighed. Cadmium was extracted by refluxing the sediments in 5 mL of hot trace-metal-grade HNO₃ for 24 h. The resulting solution was then reduced in volume to 1 mL via volatilization, and then diluted to 50 mL with deionized water. Samples were mixed and then left idle overnight to allow sediment to settle. Both aqueous Cd and sediment-Cd extracts were analyzed using a Jarrell-Ash Model 855 ICP-AES.

**Sediment Toxicity Bioassays**

*Hyalella azteca* were exposed to sediment-amended Cd at concentrations of 2, 220, 437, 646, 819, 1050 and 1400 mg kg⁻¹ (measured concentrations). Mortality data were analyzed using probit analysis to generate 10-d LC₅₀ values with 95% confidence intervals (C.I.) using SAS® software (Release 8.2, SAS Institute Inc., Cary, NC, USA). The effect of Cd on *H. azteca* growth rate was tested using one-way ANOVA calculated with SigmaStat 2.01 software (Jandel Scientific, San Rafael, CA, USA).

*Hyalella azteca* was exposed to sediment-amended Phen at concentrations that did not exceed sediment saturation, as derived by equilibrium partitioning theory (~450 mg kg⁻¹).
Phenanthrene exposure concentrations were 0, 32, 58, 116, 225, 333 mg kg\(^{-1}\) (measured concentrations). The mean mortality across sediment-Phen treatments was only 5 % ± 8, and thus, an LC\(_{50}\) for Phen could not be determined. Linear regression was used to test the relationship between Phen and mortality, and one-way ANOVA was used to test for Phen effects on growth rate.

An experiment utilizing a randomized design and 2 x 6 factorial treatment arrangement was used to test for joint-toxic interactions between Cd and Phen in sediment exposures. The design included a 0 mg kg\(^{-1}\) Phen treatment combined with 2, 216, 397, 613, 821 and 1034 mg kg\(^{-1}\) Cd and a treatment containing 144 mg kg\(^{-1}\) Phen combined with 2, 213, 275, 651, 861, and 1068 mg kg\(^{-1}\) Cd (all concentrations represent measured values). Ten-day LC\(_{50}\) values with 95% C.I.s were calculated. Due to complete mortality in the upper range of Cd-alone treatments, growth rate affects for the combined contaminants were tested using 2-way ANOVA on only the first 4 treatments comprising a 2 x 4 treatment arrangement. Water-quality measurements were collected in the Cd alone and Cd-Phen mixture treatments and compared using t-tests (or Mann-Whitney tests for data sets with non-normal distributions).

**Aqueous Joint-Toxicity Bioassay**

An experiment incorporating a randomized design with 3 x 5 factorial treatment arrangement was used to test for joint-toxic interactions between Cd and Phen in aqueous exposures. Phenanthrene treatments included 0 and 150 µg L\(^{-1}\) treatments (nominal) and an acetone carrier control. Range-finding experiments indicated that 150 µg L\(^{-1}\) Phen (nominal) caused no mortality in *H. azteca*. Phenanthrene concentrations in the 150 µg L\(^{-1}\) Phen (nominal) treatment was variable ranging from an average of 119 ± 10 µg L\(^{-1}\) upon fresh renewal, declining to 39 ± 11 µg L\(^{-1}\) prior to renewal. Phenanthrene treatments were combined with 0.0, 2.7, 5.4,
11.7, 22.7 µg L⁻¹ Cd (measured concentrations). Cadmium concentration dynamics have been observed to be less pronounced having losses of < 15% between renewals (Gust submitted). Mortality counts were taken at 24, 48 and 72 h and LC₅₀ values with 95% C.I.s were calculated. Water-quality measurements were collected for Cd alone and Cd-Phen mixture treatments and compared using Kruskal-Wallis ANOVA.

Non-overlapping 95% C.I.s were used as criteria for identifying significant differences in mortality among concentration-response curves. Effects of Cd and Phen on growth rate were tested using 2-way analysis of covariance (ANCOVA) with SAS® software, where a significant interaction term was used as criteria for interactive toxicity between the contaminants.

RESULTS

Water Quality Measures

No measured water quality parameter deviated from standards established by the US EPA (USEPA 2000) in any experiment conducted. In sediment bioassays, only dissolved oxygen differed significantly among Cd alone versus Cd-Phen mixture treatments (p = 0.034). The mean dissolved oxygen concentration for the 10-d bioassay was 6.14 mg l⁻¹ ± 0.41 for the Cd alone treatment and 5.92 mg l⁻¹ ± 0.27 for the Cd plus Phen treatment. No statistically significant differences among treatments (p > 0.05) were detected for any of the water quality parameters measured in the aqueous toxicity test.

Cd and Phenanthrene Individual Toxicities in Sediment Exposures

The resulting LC₅₀ value for H. azteca exposed to sediment-amended Cd was 484 (417-550) mg kg⁻¹ (95% C.I.s in parentheses; Figure 3.1A). Cadmium significantly affected growth rate (Figure 3.1B; one-way ANOVA, p < 0.001). Cadmium concentrations of 437 mg kg⁻¹ and above caused a significant reduction in growth rate compared to the control. (p < 0.05).
There was no relationship between Phen and mortality ($p = 0.530$, $R^2 = 0.0142$; Figure 3.2A), and mortality among all treatments was low. The highest mean mortality was observed in the control (12% ± 8), and mean mortality across all treatments was 5% ± 8. Phenanthrene significantly reduced *H. azteca* growth rate ($p = 0.007$); however, there was not a clear relationship between Phen concentration and the reduction in growth rate (Figure 3.2B). Dunnet’s multiple comparisons test ($p = 0.05$) indicated significant reductions in growth rate compared to the control in the intermediate Phen treatments (57, 116 and 225 mg kg$^{-1}$), but not at either the lowest or highest Phen concentrations (32 and 333 mg kg$^{-1}$ respectively).

**Cd and Phenanthrene Joint Toxicity in Sediment Exposures**

Although Phen did not elicit mortality, Phen increased the lethal toxicity of co-occurring Cd in sediment exposures (Figure 3.3A). In the joint-toxicity sediment bioassay, the LC$_{50}$ for sediment-amended Cd alone was 523 mg kg$^{-1}$ (461-588, 95% C.I.) which is similar to the LC$_{50}$ determined in the independent bioassay listed above. In contrast, the LC$_{50}$ determined for Cd combined with 144 mg kg$^{-1}$ of Phen was 263 mg kg$^{-1}$ (214-312, 95% C.I.). The growth-rate concentration-response for combinations of sediment-associated Cd and Phen did not differ significantly from the concentration response generated for Cd alone (Figure 3.3B). Results of ANCOVA on ranked data indicated that Cd significantly reduced *H. azteca* growth rate ($p < 0.0001$) but that Phen did not ($p = 0.0781$). No significant interaction was found among the contaminant treatments ($p = 0.2482$).

**Cd and Phenanthrene Joint Toxicity in Aqueous Exposures**

In the aqueous bioassay, Phen did not significantly increase the lethal toxicity of Cd. The concentration-response curves (not shown) and corresponding LC$_{50}$ values and 95% C.I.s (Table 3.1) were generally equivalent among the 0 µg L$^{-1}$ Phen, 150 µg L$^{-1}$ Phen and acetone carrier
Figure 3.1. Effect of sediment-amended Cd (A) and phenanthrene (B) on survivorship in *Hyalella azteca* in 10-d sediment exposures. Symbols represent means ±1 S.D. (n = 5). Cd and phenanthrene concentrations are expressed in mg kg⁻¹ dry weight sediment.
Figure 3.2. Effect of sediment-amended Cd (A) and phenanthrene (B) on growth rate in *Hyalella azteca* in 10-d sediment exposures. Points represent means ±1 S.D. (n = 5). Symbols (*) signify statistically significant differences (p < 0.05) from control as derived by Dunnett’s Test. Cd and phenanthrene concentrations are expressed in mg kg\(^{-1}\) dry weight sediment.
Figure 3.3. Effect of sediment-amended Cd-phenanthrene mixture on survivorship (A) and growth rate (B) in *Hyalella azteca* in 10-d sediment exposures. Points represent means ±1 S.D. (n = 5). In panel (B), to maintain an orthogonal data set for statistical analysis, data points within the dashed box were not used in statistical analysis (see Materials and Methods). Cd and phenanthrene concentrations are expressed in mg kg⁻¹ dry weight sediment.
Table 3.1. Aqueous Cd LC$_{50}$ values (µg L$^{-1}$) and corresponding 95% C.I.s for Cd alone, Cd including acetone carrier and Cd including phenanthrene (Phen) treatments for *H. azteca*. The target Phen concentration in the Cd & Phen treatment was 150 µg L$^{-1}$. LC$_{50}$ values are given for 24, 48 and 72h exposures. Values including asterisk (*) represent non-overlapping 95% C.I.s within a given exposure time.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24h LC$_{50}$</th>
<th>95% C.I.</th>
<th>48h LC$_{50}$</th>
<th>95% C.I.</th>
<th>72h LC$_{50}$</th>
<th>95% C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd Alone</td>
<td>10.0</td>
<td>(8.8-11.6)</td>
<td>3.5*</td>
<td>(2.9-4.2)</td>
<td>1.9</td>
<td>(1.4-2.4)</td>
</tr>
<tr>
<td>Cd &amp; Acetone</td>
<td>10.4</td>
<td>(8.9-12.2)</td>
<td>4.1</td>
<td>(3.2-5.1)</td>
<td>2.0</td>
<td>(1.5-2.5)</td>
</tr>
<tr>
<td>Cd &amp; Phen</td>
<td>12.6</td>
<td>(8.1-20.5)</td>
<td>5.3*</td>
<td>(4.4-6.4)</td>
<td>2.5</td>
<td>(1.9-3.1)</td>
</tr>
</tbody>
</table>

Control treatments for each time period. The only detectible difference among treatments involved a decrease in Cd toxicity when combined with 150 µg L$^{-1}$ Phen at the 48h time period (Table 3.1).

**DISCUSSION**

Cadmium-Phen mixtures in sediment exposures caused synergistic lethal toxicity in *Hyalella azteca* (Figure 3.3A). The 10-d LC$_{50}$ for Cd in sediment was reduced by nearly 50% from 523 mg kg$^{-1}$ (461-588, 95% C.I.) to 263 mg kg$^{-1}$ (214-312, 95% C.I.) when combined with a sublethal concentration (144 mg kg$^{-1}$) of Phen. Conversely, in aqueous exposures, there was a predominantly independent effect of Phen on Cd lethal toxicity. Although there was synergistic lethality associated with Cd-Phen mixtures in sediment, the pollutants caused an independent effect on *H. azteca* growth.

**Toxicity in Single-Compound Exposures**

The concentration of Cd required to elicit mortality in *H. azteca* was as much as 5 orders of magnitude higher in sediment than in dechlorinated tap water. These results are consistent with those found by Borgman et al. (1991). Reduced metal toxicity in the presence of sediments...
is well documented (Ankley 1994, Borgman et al. 1991). The toxicity of many metals is directly associated with the total amount of non-complexed metal found in the dissolved phase (Di Toro et al. 2001). Cadmium body burdens in amphipods have been shown to reach equilibrium within 10 d (Clason and Zauke, 2000). Based on this observation, it is presumed in the present study, *H. azteca* exposed to Cd-alone and the Cd-Phen mixture approached equilibrium tissue-Cd concentrations.

Cadmium significantly reduced *H. azteca* growth rate in sediment exposures. Reduction in *H. azteca* growth rate has previously been shown to be a sensitive indicator of Cd contamination in sediments (Milani et al. 2003). In the present study, there was no difference in endpoint sensitivity to Cd when comparing lethality and growth rate. Neither endpoint indicated a significant difference compared to the control below a Cd concentration of ~400 mg kg$^{-1}$.

Sediment-amended Phen did not elicit mortality even when concentrations approached equilibrium with the sediment organic carbon fraction (333 mg kg$^{-1}$). Similarly, greater than 90% survival occurred in *H. azteca* exposed to 1,270 nmol g$^{-1}$ (256 mg kg$^{-1}$) fluoranthene in natural-sediment exposures lasting 10 and 16 d (Driscoll et al. 1997a). In the aqueous exposures conducted in the present study, Phen ranged from 5-10% of saturation and did not elicit mortality. Lower molecular weight PAHs such as fluoranthene, anthracene and Phen are bioaccumulated quickly in *H. azteca* reaching tissue equilibrium in as little as 1 d from either sediment or aqueous exposures (Driscoll et al. 1997a, Driscoll et al. 1997b, Landrum and Scavia 1983). Therefore, in the present study, it is reasonable to assume that *H. azteca* approached steady-state Phen body concentrations during both sediment and aqueous bioassays. The tendency of *H. azteca* to quickly reach steady-state PAH body concentrations that are of relatively low magnitude may be due to its ability to efficiently metabolize and eliminate PAHs.
(Driscoll et al. 1997a, Driscoll et al. 1997b, Landrum and Scavia, 1983). Thus, *H. azteca’s* ability to regulate total PAH body burden may have contributed to the high survivorship observed in the present Phen bioassays.

Phen significantly reduced growth rate, although there did not appear to be a direct negative relationship between Phen concentration and growth. Growth rate was significantly reduced in Phen treatment concentrations above 58 mg kg\(^{-1}\) through 225 mg kg\(^{-1}\) but not at the highest treatment of 333 mg kg\(^{-1}\). Although growth rate reduction was a more sensitive indicator of sediment-Phen contamination than lethality, our results indicate that growth rate responses to Phen are inconsistent.

**Synergistic Toxicity in *Hyalaeella azteca***

Cadmium has been found to impair PAH metabolism in the estuarine fish *Fundulus heteroclitus* by inhibiting hepatic cytochrome P450 (Cyp1A) enzyme activity (van den Hurk et al. 1998a), and synergistic lethal joint-toxicity was associated with this response (van den Hurk et al. 1998b). Cytochrome P450 pathways allow organisms to biotransform, detoxify and excrete an array of organic xenobiotics including PAHs (Casarett & Doull 2001). In the present study, although Phen caused no mortality alone and appeared to increase the toxicity of Cd in Cd-Phen sediment mixtures, the synergistic toxicity observed in *H. azteca* may have been the result of Cd-mediated interference with Phen detoxification rendering Phen toxic.

George and Young (1986) showed that the PAH 3-methylcholanthrene delayed the onset of metallothionein induction in plaice, *Pleuronectes platessa*, hepatocytes by 6 d compared to plaice exposed to Cd alone. Metallothioneins are evolutionarily wide-spread, low molecular weight proteins that bind various metals *in situ* significantly reducing toxicity (Roesijadi 1992). Therefore, Phen-mediated inhibition of metallothionein induction in *H. azteca* could have increased the lethality in *H. azteca* exposed to the Cd-Phen mixture.
In sediments, biotic exposure to metals may be altered by PAH. Millward et al. (2004) found increased sediment retention of Cu and Cr in flow-through microcosms when diesel fuel was a co-contaminant. The differential retention/flux and/or bioavailability of contaminants caused by co-contaminants may alter exposure to and hence the overall toxicity to organisms in sediment environments. Little research has been conducted examining the effect of PAH on the bioavailability of metals. However results in related research employing sediment bioassays identical to those used in this study (Gust submitted) indicated that Phen does not alter Cd concentrations found in the dissolved phase (the most bioavailable metal fraction). The results of Gust (submitted) also indicate that the observed increase in \textit{H. azteca} mortality when exposed to Cd-Phen mixtures in sediment is independent of the Cd concentration dissolved in overlying water.

Even though the bioavailability of Cd may not be altered by Phen, modifications in animal behavior or physiology may alter metal exposure and bioaccumulation (Gust and Fleeger in preparation). Behavioral or physiological responses of \textit{H. azteca} to sediment-associated Phen may have increased Cd bioaccumulation and overall toxicity via mechanisms that are not manifested in aqueous exposures. For example, Fair and Sick (1983) observed that the Black Sea Bass, \textit{Centropristis striata}, assimilated higher concentrations of Cd in various tissues from food containing Cd and naphthalene compared to food containing Cd alone. Recently conducted experiments with \textit{H. azteca} indicate Phen increased Cd bioaccumulation rate in sediment exposures, but not in water-only exposures (Gust submitted). In the present study, the observed increase in \textit{H. azteca} mortality in sediment exposures, but not in aqueous exposures, may have resulted from Phen-mediated increases in Cd bioaccumulation rate.

It has been suggested that metals bioaccumulate and exert toxicity in \textit{H. azteca} primarily from exposure to overlying water associated with contaminated sediment (Borgmann et al. 2001,
Borgman and Norwood 1999, Warren et al. 1998). However, the critical body residue of Cd has been shown to be 2-2.6 fold higher when accumulated from sediment than from aqueous sources (Borgmann et al. 1991). This indicates that sediments may provide an alternative source for Cd uptake that may not reach the site of toxic action. Deposit-feeding organisms have been shown to bioaccumulate the majority of their metal body burden through ingestion of metal-contaminated sediment (Wang and Fisher 1999). Furthermore, it seems logical that ingestion of metal-contaminated sediment could lead to differential exposure among tissues compared to uptake from aqueous sources. These observations indicate synergistic toxicity between Cd and Phen may occur if Phen enhances the partitioning of Cd to the site of toxic action, or if Phen renders Cd toxic to tissues typically unassociated with Cd toxicity. Autoradiography with radioactive Cd would allow visualization of the location of metals to determine if sediment exposure alters the distribution of Cd among tissues (Rouleau et al. 2001).

Concentration-dependent interactions in which synergisms and antagonisms occur at different exposure concentrations of metals and PAH have been documented (Moreau et al. 1999, van den Hurk et al. 1998b), and may potentially explain the differences in results between sediment and aqueous exposures. In both the sediment- and aqueous-toxicity bioassays in the present study, Cd concentrations ranged from those causing low to complete mortality, and all Phen concentrations were sublethal. Based on the equilibrium partitioning coefficient for Phen in the test sediment, the dissolved Phen concentration found in porewater of the sediment bioassay is comparable to that used in the aqueous bioassay within a factor of two. Therefore, exposure concentrations used in both the sediment and aqueous tests were similar indicating that the differential toxicity found between these bioassays is most likely not the result of a concentration-dependent interaction between Cd and Phen.
Although synergistic lethal effects were found between Cd and Phen in sediment bioassays, the effect of the combined contaminants was independent for growth rate. This observation brings into question the choice of appropriate experimental endpoints when assessing environmental risk. Sub-lethal endpoints, including growth rate, may be considered more sensitive indicators of sediment toxicity than lethality. This study indicates growth rate was not more sensitive than lethality for Cd or Phen toxicity. Additionally, growth rate was not indicative of the deleterious synergistic lethal toxicity found between these contaminants. Growth rate of *H. azteca* may not be an appropriate endpoint in acute toxicity bioassays.

**Implications**

Based on current hypotheses in chemical-mixture toxicology, dissimilar contaminants are expected to have independent joint toxicity (Price et al. 2002, Broderius 1991). Metals and PAH represent different chemical classes that exhibit dissimilar toxicology and typically have unrelated environmental chemistries. The results of the present study show that the assumption of independent toxicity between these chemical classes cannot be made with confidence. Additionally, the results of this study as well as examples from the literature indicate that joint-toxic interactions between metals and PAH are possible if not probable. These observations call into question the accuracy of risk assessment protocols employing independent or additive toxicity as their underlying assumptions for metal-PAH contaminated environments. As a broader implication of these results, chemical toxicity protocols should include testing of combined contaminants when the joint toxicity of chemical classes in a given mixture is unknown.

The joint-toxic effects of metals and PAHs are potentially species-specific (Lemaire-Gony and Lemaire 1992, George and Young 1986), and as demonstrated by the present study,
metal-PAH toxicity may be endpoint specific and dependent on the environmental-exposure media. As an implication of these results, the interpretation of standardized toxicity bioassays used to assess environmental quality (including whole effluent toxicity (WET) tests and single compound toxicity tests) must be made with caution. For example, increased mortality was observed in *H. azteca* exposed directly to field-collected sediment containing a mixture of metals, PAH and PCBs compared to those exposed only to the overlying water (Ingersoll et al 2000). This demonstrates that the assessment protocols mentioned above may underestimate potentially hazardous contaminant-mixture effects representative of complex sediment environments. Improved understanding of the environment-specific mechanisms influencing interactive toxicity between metals and PAH is required for development of a more predictive global model of metal-PAH toxicity.

**LITERATURE CITED**


George SG, Young P (1986) The time course of effects of cadmium and 3-methylcholanthrene on activities of enzymes of xenobiotic metabolism and metallothionein levels in the plaice, Pleuronectes platessa. Comparative Biochem Physiol 83C:37-44

Gust KA (Submitted) Exposure-related effects on Cd bioaccumulation explains toxicity of Cd-phenanthrene mixtures in Hyalella azteca. Environ Toxicol Chem

Gust KA, Fleeger JW (Submitted) Exposure to cadmium-phenanthrene mixtures elicits complex toxic responses in the freshwater tubificid oligochaete, Ilyodrilus templetoni. Arch Environ Contam Toxicol


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Moreau CJ, Klerks PL, Haas CN (1999) Interaction between phenanthrene and zinc in their toxicity to the sheepshead minnow (*Cyprinodon variegatus*). Arch Environ Contam Toxicol 37:251-257


Sanger DM, Holland AF, Scott GI (1999a) Tidal creek and salt marsh sediments in South Carolina coastal estuaries: I. Distribution of trace metals. *Arch Environ Contam Toxicol* 37:455-457


CHAPTER 4

EXPOSURE-RELATED EFFECTS ON CADMIUM BIOACCUMULATION EXPLAINS TOXICITY OF CADMIUM-PHENANTHRENE MIXTURES IN *HYLELLA AZTECA*
INTRODUCTION

In classical toxicology, combined doses of chemicals with dissimilar modes of toxic action are hypothesized to elicit independent toxicological effects (Cassee et al. 1998). Although this hypothesis accurately characterizes the joint toxicity of some classes of dissimilar compounds (Altenburger et al. 2004), it may be inaccurate for metal-PAH combinations (van den Hurk et al. 1998, Viarengo et al. 1997, Bruschweiler et al. 1996, Fair 1986, George and Young 1986, Forlin et al. 1986, Eaton et al. 1980). In addition to toxicological interactions, factors related to environmental exposure may also contribute to “apparent” interactive effects. Apparent interactions occur when synergistic or antagonistic joint-toxic effects are manifested in organisms, but are not the result of interactive toxicological mechanisms. For example, environment type (Millward et al. 2004, Gust 2005, Chapters 5 and 6) and route of exposure (Gust 2005, Fair and Sick 1983, Chapters 5 and 6) have been observed to contribute to “apparent” interactive effects between metals and PAHs. Much emphasis in ecotoxicology has been placed on understanding how organisms are exposed to contaminants and how exposure affects bioaccumulation and resultant toxicity. Toxicity of contaminant mixtures to organisms in natural environments will invariably be related to both exposure-mediated effects and the toxicological interplay of bioaccumulated mixture components. In the present study, exposure-mediated effects are investigated.

The bioavailability of both metals and PAHs are each strongly influenced by the physico-chemical properties of sediments in aquatic environments (Chapman et al. 1998, Di Toro et al. 1991). Although the co-occurrence of these contaminants in urban sediments is suggested to be prevalent (Callender and Rice 2000, Van Metre et al. 2000) little is known about their combined effects in sediment-dwelling organisms. Mixture effects in sediments are especially complex for
deposit-feeding animals that are exposed to both dissolved contaminants and contaminated food / sediment. Contaminant bioavailability and organismal exposure strongly influence both metal and PAH bioaccumulation (Di Toro and McGrath 2000, Wang and Fisher 1999) which can directly affect toxicity (Barron et al. 2002). Prior to the present study, the occurrence of mixture-mediated alterations in contaminant bioavailability and organismal exposure in sediment exposures was untested.

In a previous investigation of metal-PAH mixture effects in H. azteca, sublethal concentrations of Phen combined with Cd elicited significantly higher lethality than Cd alone in sediment exposures, and had no affect on the lethal toxicity of Cd in water-only exposures (Gust 2005). Because the Cd-Phen mixture elicited independent effects in aqueous exposures, the apparent synergism observed in sediment bioassays was unlikely the result of true toxicological interaction. The purpose of the present study was to elucidate the cause of this apparent synergism. Sediment-uptake, water-only uptake and elimination-rate kinetics bioassays were conducted to determine if Phen altered Cd bioaccumulation rates in H. azteca. Additionally, a sediment bioassay was conducted to investigate Phen effects on dissolved Cd concentrations in overlying water and water quality parameters that influence Cd bioavailability. We hypothesized that Phen would have no affect on parameters associated with Cd bioavailability and no affect on Cd uptake / elimination kinetics regardless of exposure-source.

**MATERIALS AND METHODS**

Bioassays were conducted using or extending U.S. Environmental Protection Agency method 100.1 for Hyalella azteca (U.S. Environmental Protection Agency 2000). Sub-adults (2-3 weeks old) were collected as described by Driscoll et al. (1997) then acclimated to test conditions for 1 day. Exposure chambers were maintained at 23 ± 1°C with a 16L:8D
photoperiod. Amphipods were fed 0.15 mL individual⁻¹ aliquots of yeast, Cerophyl® and trout chow (U.S. Environmental Protection Agency 2000) daily, in all bioassays. Static-renewal water-replacement procedures were employed where the entire water volume was replaced daily. After collection, amphipods were transferred to clean water and allowed to depurate gut contents for 6 h. Amphipods were then oven dried at 70°C for 24h, and dry weights determined. Information describing *H. azteca* culturing techniques, methods for recovering animals from bioassays and enumeration protocols can be found in Gust (2005).

Sediment characteristics, Phen-spiking procedures and analytical procedures for determining Phen concentrations in sediment are described in Gust (2005). Cadmium chloride (98% purity, Sigma Chemical Co.) was dissolved in deionized water and slowly amended to a specific mass of wet sediment (as determined by sediment wet:dry ratio) to achieve a targeted sediment concentration. In the sediment-Cd uptake experiment, $^{109}$Cd (PerkinElmer Life Sciences, Inc.) was included in the metal-spike solution targeting an activity of 300 counts per minute (cpm) mg⁻¹ dry weight sediment (150 µCi total). Each Cd solution was added dropwise to sediment in plastic jars undergoing mixing with a handheld mixer. For joint-toxicity bioassays, Phen was amended to sediment prior to addition of Cd. Day-0 sediment Cd concentrations were analyzed by inductively-coupled argon plasma mass spectrometry (ICP-MS) using a PerkinElmer Sciex, Elan 9000 ICP-MS (PerkinElmer Sciex, Inc.), and $^{109}$Cd activities in sediment, *H. azteca* tissues and overlying water were measured using a Perkin Elmer / Wallac Wizard 1470 gamma counter. For non-radioactive Cd analysis, two replicates of each Cd treatment were freeze-dried, milled and weighed. Cd was extracted into 10 mL of trace-metal-grade HNO₃ using a Perkin Elmer/Anton Paar, Multiwave 3000, microwave sample preparation system (USEPA 1986). The sediment-extract was poured into a 50 mL plastic centrifuge tube
through Whatman ashless filter paper. The filter was rinsed 3 times with 3% trace-metal-grade HNO₃ to a volume of 50 mL. This solution was diluted to a concentration within the linear detection limits of ICP-MS. Standard reference material extractions and procedural blanks were included for quality control purposes.

*Hyalella azteca* was exposed to sediment treatments including Cd alone (123.2 ± 2.1 mg kg⁻¹) and Cd (135.7 ± 2.6 mg kg⁻¹) combined with Phen (133.0 ± 5.1 mg kg⁻¹). Cd body burdens were determined for animals exposed to each treatment after 1, 3, 6 and 10 d of exposure. The experimental design was randomized including a 2 x 4 factorial treatment arrangement. Ten randomly-selected individuals were exposed in 5 replicate 100-mL plastic beakers containing 20 mL test sediment and 80 mL overlying dechlorinated tap water. Animals were collected from each exposure chamber, enumerated and rinsed three times with deionized (D.I.) water. Amphipods were allowed to depurate gut contents for 6 h, rinsed, transferred to clean vials and gamma counted.

In the sediment-Cd uptake experiment, the percent increase in individual *H. azteca* dry weight was quantified for each contaminant treatment at each exposure period. The mean dry weight individual⁻¹ for 5 groups of 10 randomly selected animals collected at experiment initiation was subtracted from the dry weight individual⁻¹ for each replicate. The effects of Cd alone and the Cd-Phen mixture on whole-amphipod dry weight were compared over time using 2-way ANOVA (SigmaStat 2.01, Jandel Scientific).

Fecal pellets deposited during gut clearance for d 3, 6 and 10 were collected from each replicate, transferred to a clean vial and gamma counted. Count times were adjusted to ensure < 5% counting error. Whole-amphipod and fecal dry weights were measured for each replicate after gamma counting. Counts per minute data were corrected for radioactive decay and for
gamma-counting efficiency to calculate disintegrations per minute (dpm). The mean activity of 
\(^{109}\text{Cd}\) in test sediment was determined as dpm mg\(^{-1}\) dry weight sediment (1207 ± 19 dpm mg\(^{-1}\) for Cd alone and 1259 ± 23 dpm mg\(^{-1}\) for the Cd-Phen mixture, \(n = 4\) for each). The ratio of the measured sediment-Cd concentration to dpm mg\(^{-1}\) was used to determine the dry-weight concentration of Cd in \(H.\ azteca\) tissue and feces. The effect of Phen and exposure time on Cd body burden, growth, Cd concentration in feces and total fecal production were tested using 2-way ANOVA. A Michaelis-Menten one-site saturation model was used to fit the kinetic uptake data and to calculate \(B_{\text{max}}\) and 95% C.I. for each treatment using SigmaPlot\textsuperscript{®} 8.02 (SPSS Inc.). Non-overlapping 95% confidence intervals were used as criteria for identifying significant differences in \(B_{\text{max}}\) values between Cd alone and Cd including Phen.

Because Cd is thought to bioaccumulate predominantly from overlying water in sediment exposures (Warren et al. 1998, Borgmann et al. 1991), an experiment was conducted to test for Phen effects on dissolved Cd concentrations in overlying water. Two Cd treatments (300 and 500 mg kg\(^{-1}\) dry weight sediment targets, measured Cd concentrations in Table 4.1) with and without a sublethal concentration of Phen (124 mg kg\(^{-1}\), measured concentration) were examined. Ten randomly selected \(H.\ azteca\) were exposed in 4 replicate 400-mL plastic beakers containing 100 mL test sediment and 200 mL overlying dechlorinated tap water. On d-10, amphipods were recovered, enumerated and percentage mortality calculated. Two-way ANOVA was used to test for treatment effects on mortality. During the 10 d bioassay, initial (d-0), d-5 and d-10 overlying water samples were taken from all replicates for Cd and Phen analysis. Non-dissolved material was removed by filtration through 0.45 µm membrane filters (Pall Life Sciences). Cadmium samples were acidified with trace-metal grade HNO\(_3\) and stored at 4°C for less than 21 d prior to ICP-MS analysis. Phenanthrene samples were stored in the dark at 4°C and analyzed by HPLC.
Table 4.1. Sediment bioassay results and parameters. Treatments included 2 levels of Cd (targets 300 and 500 mg kg⁻¹ dry wt. sediment) with and without a sublethal concentration (124 mg kg⁻¹) of phenanthrene (Phen). The top panel provides the bulk-sediment Cd concentrations for each treatment at d-0 in mg kg⁻¹ (mean ± 1 S.D., n = 2), dissolved Cd concentration in overlying water at days 0, 5 and 10 in µg L⁻¹ (mean ± 1 S.D., n = 4) and *H. azteca* 10 d mortality (mean ± 1 S.D., n = 4). The affect of Phen on Cd concentrations in overlying water and mortality were tested using 3- and 2-Way ANOVA respectively. The bottom panels provide pH and dissolved oxygen values measured in overlying water for Cd alone and Cd-Phen mixture treatments (mean ± 1 S.D., n = 4). T-tests or Man-Whitney ranked sum tests were used to test treatment effects on pH and 2-way ANOVA was used to test Phen effects (including time) on dissolved oxygen.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Sediment Conc. (mg kg⁻¹)</th>
<th>Overlying Water Conc. (µg L⁻¹)</th>
<th>% Mortality at Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 5</td>
</tr>
<tr>
<td>300 mg kg⁻¹ Cd</td>
<td>292 ± 19</td>
<td>2.6 ± 0.5</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>300 mg kg⁻¹ Cd &amp; 124 mg kg⁻¹ Phen</td>
<td>328 ± 8</td>
<td>2.6 ± 1.0</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>P Value</td>
<td></td>
<td>P &gt; 0.05ᵃ</td>
<td>P &gt; 0.05ᵃ</td>
</tr>
<tr>
<td>500 mg kg⁻¹ Cd</td>
<td>503 ± 4</td>
<td>4.3 ± 0.2</td>
<td>4.4 ± 1.1</td>
</tr>
<tr>
<td>500 mg kg⁻¹ Cd &amp; 124 mg kg⁻¹ Phen</td>
<td>500 ± 8</td>
<td>4.8 ± 0.2</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>P Value</td>
<td></td>
<td>P &gt; 0.05ᵃ</td>
<td>P &gt; 0.05ᵃ</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Overlying Water pH</th>
<th>Day 0</th>
<th>Day 5</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd Alone</td>
<td>--</td>
<td>7.54 ± 0.01</td>
<td>7.85 ± 0.08</td>
</tr>
<tr>
<td>Cd &amp; 124 mg kg⁻¹ Phen</td>
<td>--</td>
<td>7.55 ± 0.01</td>
<td>7.79 ± 0.05</td>
</tr>
<tr>
<td>P Value</td>
<td>P = 0.437</td>
<td>P = 0.140</td>
<td>P = 0.934</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dissolved Oxygen (% Saturation)</th>
<th>Day 0</th>
<th>Day 5</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd Alone</td>
<td>--</td>
<td>71.3 ± 1.7</td>
<td>61.0 ± 7.7</td>
</tr>
<tr>
<td>Cd &amp; 124 mg kg⁻¹ Phen</td>
<td>--</td>
<td>70.3 ± 2.1</td>
<td>56.0 ± 2.8</td>
</tr>
<tr>
<td>P Value</td>
<td>P &gt; 0.05ᶜ</td>
<td>P &gt; 0.05ᶜ</td>
<td>P &gt; 0.05ᶜ</td>
</tr>
</tbody>
</table>

ᵃ Main effects of Phen, time and their interaction on dissolved Cd concentration were not significant. A significant interaction occurred between Cd and Time (P = 0.013).

ᵇ Main effect of the sublethal 124 mg kg⁻¹ Phen treatment combined with Cd on *H. azteca* mortality.

ᶜ Main effects of Phen and time on dissolved oxygen concentration.

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within 3 d of collection. The effects of sediment-Cd concentration, the presence of Phen, time and their interactions on dissolved Cd concentration in overlying water were analyzed by 3-way ANOVA (SAS® software, Release 8.2) after natural-log transformation.

An aqueous uptake bioassay was conducted exposing 100 randomly selected *H. azteca* in 1 L glass beakers containing 600 mL of $^{109}$Cd-labelled treatment solution (60 µCi total). Treatments included Cd alone, Cd including Phen (100 µg L$^{-1}$ Phen target, see Figure 4.1A for measured concentrations) and Cd including an acetone carrier (0.1 ml L$^{-1}$ in exposure medium) each with 4 replicates. Phenanthrene required dissolution in acetone, therefore a control for acetone effects was required. Equivalent $^{109}$Cd concentrations were targeted in all treatments without the addition of non-radioactive Cd. Each treatment solution was prepared no more than 1h prior to daily water renewal. Dissolved Cd and Phen concentrations were measured in each replicate for each treatment prior to and after each renewal. Cadmium concentrations were calculated using the specific activity of $^{109}$Cd to determine $^{109}$Cd mass as a function of the dpm L$^{-1}$ of test solution (see Figure 4.1B for measured concentrations). Phenanthrene concentrations were determined by HPLC (Figure 4.1A). The effect of the exposure treatments on Cd concentration in aqueous exposure media was tested using Kruskal-Wallis ANOVA. *H. azteca* was exposed to the 3 treatment solutions for 6, 12, 24, 48, 96 or 192 h. At each time, 10 randomly selected amphipods were removed from each replicate and rinsed 3 times in D.I. water. They were then transferred to clean vials and gamma counted. Cadmium body concentrations in *H. azteca* were calculated using the specific activity of $^{109}$Cd to determine $^{109}$Cd weight as a function of the dpm mg$^{-1}$ dry weight tissue (µg g$^{-1}$). Uptake rate constants from the dissolved phase ($k_u$) were calculated from linear regression ($k_u = L g^{-1} d^{-1}$).
Figure 4.1. Dynamics in phenanthrene (A) and Cd (B) concentrations over time during the aqueous Cd uptake experiment. Measurements taken before and after daily-water renewal. Phenanthrene (Phen) concentrations determined by HPLC and Cd concentrations determined based on the specific activity of $^{109}$Cd (see methods for calculations). Symbols represent means ± 1 S.D. (n = 4).
In the elimination experiment, *H. azteca* was exposed for 192h, collected, processed and tissue radioactivity counted as described above. Amphipods were then transferred to 1-L depuration chambers in which dechlorinated-tap water was renewed daily. Amphipods were rinsed 3 times in D.I. water and gamma counted at 12, 24, 48, 96 and 192 h. Percentage elimination was calculated for each replicate by dividing the dpm at each time point by the initial dpm. Effects of Phen and exposure time on Cd uptake and elimination kinetics were tested using 2-way repeated-measures ANOVA. Effects of the acetone control were also tested in this fashion. Elimination rate constants (ke) were determined using slope values from linear regression (ke = % elimination d⁻¹).

Water quality parameters were measured for each contaminant treatment in all bioassays. Dissolved oxygen concentration and temperature were measured daily in overlying water of 4-5 randomly selected replicates (depending on experiment) using an Orion model 820 oxygen meter. Overlying-water pH and ammonia concentrations were measured at experiment initiation and termination in 4-5 randomly selected replicates with an Oakton® Ion 510 Series pH meter and a NH₃/NH₄ aquarium test kit (Aquarium Pharmaceuticals, Inc.). Comparison of contaminant treatment effects (Cd alone, Cd including Phen and, if applicable, Cd including the acetone control) and the effect of time on both the dissolved oxygen concentration and overlying water temperature were analyzed using 2-way ANOVA (data not meeting assumptions of ANOVA were rank transformed). Mann-Whitney ranked sum tests or t-tests were used to test treatment effects on pH and ammonia concentration (initial and final) in Cd-bioavailability and sediment-Cd uptake bioassays. ANOVA was used to test for treatment effects on pH and ammonia concentration in aqueous-Cd uptake and elimination bioassays.
RESULTS

Water quality parameters deviated from standards established by the USEPA (2000) only in the aqueous uptake bioassay in which dissolved oxygen fell below 40% saturation after 96h in treatments containing acetone. Data from treatments that did not meet USEPA standards were excluded from analyses. The only significant difference (P < 0.05) in water quality values detected among contaminant treatments (Cd alone, Cd including Phen and/or Cd including acetone carrier) occurred in the sediment-Cd uptake bioassay. In that bioassay, a difference in temperature was detected, though the mean temperatures for Cd alone and Cd including Phen treatments were nearly indistinguishable (22.6°C ± 0.2 and 22.6 ºC ± 0.1 respectively). No interactive effects with time (P < 0.05) were observed in any bioassay.

Sediment Exposures

The presence of Phen increased Cd uptake from sediment into \textit{H. azteca} tissues over time (Figure 4.2). Cadmium body burdens for individuals exposed to the Cd-Phen mixture were 3.6x higher than those exposed to Cd alone by d-10. Phenanthrene, exposure period and their interaction significantly affected Cd body burden (P < 0.001, for each). Cadmium uptake-rate kinetics for amphipods exposed to Cd alone and the Cd-Phen mixture were indistinguishable at d-1 (P = 0.701). After d-1, Cd body burdens increased greatly in amphipods exposed to the Cd-Phen mixture. The projected $B_{\text{max}}$ for the Cd alone treatment was 47.2 µg g$^{-1}$ (36.2-58.3, 95% C.I.) whereas, the $B_{\text{max}}$ for Cd in the Cd-Phen mixture was 221.1 µg g$^{-1}$ (117.8-324.3, 95% C.I.). Growth rate increased linearly over time through d-10 but was not effected by the presence of Phen (P = 0.092, Figure 4.2B).

Phenanthrene did not affect the mass of feces deposited during depuration and had no effect on Cd concentration in feces (P = 0.654 and 0.819 respectively, Figure 4.3). The mass of depurated fecal material decreased over time (P = 0.002). There was no difference in the Cd
Figure 4.2. Kinetic uptake of Cd into *H. azteca* tissues (A) and *H. azteca* growth rate (B) when exposed to Cd alone and the Cd-phenanthrene mixture in sediments. Phen = Phenanthrene. Measured Cd concentrations in Cd alone and Cd-Phen mixture treatments were 123.2 ± 2.1 mg kg⁻¹ and 135.7 ± 2.6 mg kg⁻¹ respectively. Symbols represent means ± 1 S.D. (n = 5). The Michaelis-Menton 1-site saturation model was used to fit uptake kinetics data and linear regression was used to fit growth data. Body burdens and growth rates were measured as µg Cd g⁻¹ dry body weight and % increase in body dry weight individual⁻¹ over time respectively.
concentration in feces among time periods (P = 0.096). Dry weight of feces ranged from 4-6% of whole animal dry weight. Cadmium concentrations in feces were generally higher than in bulk sediment: 129.5 ± 7.5 (n = 4) and 191.8 ± 56.8 (n = 30) mg kg⁻¹ respectively.

The difference between initial sediment-Cd concentrations in Cd alone and Cd-Phen mixture treatments was small (Table 4.1); therefore, these treatments were considered equivalent for subsequent analyses. The results of 3-way ANOVA indicated Phen had no effect (P = 0.912) on dissolved Cd concentration in overlying water. Additionally, water quality analyses indicated no difference in overlying water pH, regardless of the presence of Phen, at experiment initiation (d-0), d-5 or d-10. An increase in sediment-Cd concentration from 300 to 500 mg kg⁻¹ (target) significantly increased (P < 0.001) the concentration of Cd in the dissolved phase. In general, dissolved Cd concentrations in overlying water increased proportionally with increases in sediment concentration, regardless of the presence of Phen. Dissolved Cd concentrations tended to increase slightly with time, but at d-10, the dissolved Cd concentration for the Cd-Phen mixture treatment containing 500 mg kg⁻¹ Cd decreased appreciably. A significant interaction between Cd and time (P = 0.013) was detected and likely resulted from this effect. Mortality was higher in treatments containing the Cd-Phen mixture than in treatments containing Cd alone (Table 4.1), which is consistent with previous findings (Gust 2004). A test of Cd effects on Phen bioavailability could not be conducted because dissolved Phen concentrations in overlying water were below detection limits of HPLC for all treatments.

**Water-Only Exposures**

Both Cd and Phen concentrations in water-only exposures were variable over the time-course of the aqueous uptake experiment (Figure 4.1). Losses of Phen in water-only exposures exceeded 50% between daily water renewals (Figure 4.1A). Variability in Cd concentrations
Figure 4.3. The effect of Cd alone and the Cd-phenanthrene mixture on fecal mass (A) and Cd concentration in feces (B) in sediment exposures. Phen = Phenanthrene. Measured Cd concentrations in Cd alone and the Cd-Phen mixture treatments were 123.2 ± 2.1 mg kg\(^{-1}\) and 135.7 ± 2.6 mg kg\(^{-1}\) respectively. Bars represent means ± 1 S.D. (n = 5).
were less pronounced between water renewals with a mean loss of 13.0% ± 8.5 (n = 24) across exposure treatments daily (Figure 4.1B). Neither Phen nor the acetone control affected the overall concentration of Cd in the treatment solutions (P > 0.05). Cadmium uptake rate constants from the dissolved phase (k_{u}) for Cd alone and the Cd-Phen mixture were 13.6 and 13.0 L g⁻¹ d⁻¹ respectively.

Cadmium uptake kinetics were generally equivalent for amphipods exposed to Cd alone and the Cd-Phen mixture in aqueous exposures (Figure 4.4A). Data for the 192-h exposure period were excluded from analysis because water quality parameters did not meet USEPA standards (USEPA 2000). Cadmium body burdens increased steadily through 96 h in all treatments and did not appear to approach equilibrium (Figure 4.4A). The only significant difference in Cd uptake associated with contaminant treatments occurred between Cd-Phen mixture treatment and the Cd-acetone procedural control.

Cadmium elimination in *H. azteca* did not differ among individuals exposed to Cd alone and the Cd-Phen mixture (Figure 4.4B). Repeated measures ANOVAs suggested that there was no difference in elimination kinetics among animals exposed to Cd alone and the Cd-Phen mixture (P = 0.226) or Cd alone and Cd-acetone control (P = 0.603). Cadmium elimination rates (k_{ew}) for Cd alone and the Cd-Phen mixture were 3.8% and 2.9% total body burden d⁻¹ respectively.

**DISCUSSION**

Sublethal concentrations of Phen combined with Cd increased lethality in sediment exposures, but not in water-only exposures in *H. azteca* (Gust 2005). This suggests the observed synergistic toxicity was not the result of a toxicological interaction, but an interaction mediated by environmental exposure. Corresponding with observed lethal effects, Phen increased Cd
Figure 4.4. Kinetic uptake (A) and elimination (B) of Cd into/out of *H. azteca* tissues in aqueous exposures. *H. azteca* were exposed to equivalent concentrations of $^{109}$Cd either alone or in combination with phenanthrene (Phen) (100µg L$^{-1}$ target). A procedural control was included to test for effects of the acetone carrier used to amend Phen into treatment solutions. Symbols represent means ± 1 S.D. (n = 4). Elimination data were fit using linear regression. See Figure 4.1 for measured Cd and Phen exposure concentrations.
bioaccumulation rate in sediment exposures but had no affect on Cd bioaccumulation in water-only exposures. In sediments, Phen caused no change in dissolved Cd concentration or in water-quality parameters that influence Cd bioavailability. It is therefore likely that increased Cd bioaccumulation is responsible for increased lethality in Cd-Phen mixtures and that the increase was, in some way, associated with feeding or digestive processes. Details and rationale for these conclusions are discussed below.

**Effect of Phenanthrene on Cd Bioaccumulation and Bioavailability**

In sediment exposures, Phen increased Cd bioaccumulation rate, total Cd body burden and increased projected equilibrium tissue-Cd concentrations ($B_{\text{max}}$) by nearly 5x, from 47.2 (36.2-58.3, 95% C.I.) to 221.1 µg g$^{-1}$ (117.8-324.3, 95% C.I.), in *Hyalella azteca* (Figure 4.2A). No previous study has investigated effects of PAH on metal bioaccumulation from sediments, but PAH-mediated increases in Cd bioaccumulation from dietary sources have been documented (Fair & Sick 1983). In the present study, Phen had no effect on *H. azteca* growth rate in sediment bioassays (Figure 4.2B). Growth can dilute contaminant body burdens by decreasing the ratio of bioaccumulated contaminant to total body weight (Wang and Fisher 1999). Results of the present study indicate increased Cd bioaccumulation observed in sediment exposures is not growth-related. Although Phen effects on Cd binding to the exoskeleton and Cd retention in the gut were not specifically quantified, a thorough rinsing protocol was used to minimize Cd association with the exoskeleton and a 6-h gut clearance period was employed to maximize sediment depuration. Furthermore, Cd bound to exoskeleton and/or non-assimilated Cd in the gut would not likely contribute to the increased lethality observed in *H. azteca* exposed to the mixture of Cd and Phen in sediment.

Sublethal concentrations of Phen had no effect on the lethal toxicity of Cd (Gust 2004), Cd bioaccumulation kinetics (Figure 4.4A), or Cd elimination kinetics (Figure 4.4B) in water-
only exposures. Studies investigating affects of organic contaminants on metal bioaccumulation in aqueous exposures have provided mixed results. Phenanthrene did not affect Cd bioaccumulation in the marine mussel *Mytilus galloprovincialis* (Viarengo et al. 1987), had no effect on Cu bioaccumulation or elimination in the marine mussel *Mytilus edulis* (Moore et al. 1984), reduced Zn bioaccumulation in sheepshead minnow (*Cyprinodon variegates*) (Moreau et al. 1999) and the pesticide chlorpyrifos caused increased rates of methyl-mercury bioaccumulation in *H. azteca* (Steevens and Benson 2001). It is difficult to make general conclusions about how organic contaminants affect metal bioaccumulation and elimination because data are sparse, and more importantly, no established experimental protocols have been developed to compare results among experiments. Without the ability to integrate data among a diversity of investigations, the already complex science of mixture ecotoxicology will continue to proceed slowly and contribute only marginally to environmental quality assessment. We suggest standardized bioassays be employed when investigating mixture effects.

The relationship between total metal body burden and toxicity may not be directly related in invertebrates. Bioaccumulated metals may be metabolically active and contribute to toxicity or they may be bound to a terminal ligand rendering the metal non-toxic (Rainbow 2002). Mechanisms of biological metal detoxification in invertebrates include binding of metals to metallothioneins and sequestration into metal-rich granules (MRG) (Rainbow 2002). Metal bioaccumulation rate has been demonstrated to be more indicative of toxicity than total metal accumulation (Barron et al. 2002). If the rate of metal detoxification occurs more slowly than metal bioaccumulation rate, toxicity may be induced. Partitioning of Cd to metallothionein was unaffected by Phen in *M. galloprovincialis* (Viarengo et al. 1987) and PAH delayed metallothionein induction in the marine fish *Pleuronectes platessa* (George and Young 1986).
Unless Phen enhanced metal detoxification mechanisms in *H. azteca*, the Phen-induced increase in Cd bioaccumulation rate likely resulted in increased concentrations of metabolically active Cd in tissues, and thus increased lethality.

Although Phen greatly increased Cd body burdens in *H. azteca* exposed to contaminated sediment, Phen did not affect parameters that influence Cd bioavailability. Redox potential gradients in sediments control biogeochemical reactions influencing the quantity of metal that partitions into the dissolved phase (Chapman et al. 1998). Oxygen concentrations in overlying water strongly influences redox-potential gradients in sediments, especially at the sediment-water interface where the majority of metal exchange occurs (Lee et al. 2000). Dissolved oxygen concentrations among all sediment bioassays in the present study and in Gust (2005) were equivalent. Parallel studies using the same sediment and sediment-preparation techniques indicated that Phen had no effect on acid volatile sulfide (AVS) or simultaneously extractable metal (SEM) concentrations (Chapter 6). Therefore, Cd partitioning into the aqueous phase is not likely affected by Phen. The most-bioavailable fraction of Cd found in the dissolved phase occurs as the free ion Cd$^{+2}$ (Chapman et al. 1998). Ions that compete with Cd$^{+2}$ for the biotic ligand (i.e. H$^+$, Ca$^{+2}$ and Na$^+$) as well as organic (i.e. DOC) and inorganic (i.e. Cd OH$^+$, Cd CO$_3^{+}$ and Cd Cl$^+$) ligand complexes that reduce Cd$^{+2}$ concentrations in solution (Di Toro et al. 2001) also affect Cd toxicity. Phenanthrene has no clear association with either the “competing” or “complexing” inorganic ions. Because Phen is a non-ionic PAH, Cd$^{+2}$ should have a low affinity for Phen. This suggests Phen is unlikely to act as an organic ligand for Cd. Additionally, dissolved Phen concentrations in overlying water were low, further reducing the probability of association between Cd and Phen molecules in solution. The percentage of dissolved metal contributing to the free ion phase is also influenced by pH (Riba et al. 2003, Erickson et al.)
Analysis of water quality parameters for all bioassays in this study and in Gust (2005) have consistently indicated no difference in pH among Cd alone and Cd-Phen mixture treatments (Table 4.1). Based on these results, it is unlikely that Phen altered *H. azteca*’s exposure to Cd from the dissolved phase.

**Feeding Effects on Cd Bioaccumulation**

Although *H. azteca* was given daily meals of non-contaminated food, their feces contained greater concentrations of Cd than bulk sediment. Cadmium partitions strongly to natural organic material (humus) in sediments (Besser et al. 2003), and deposit feeders have been observed to selectively ingest these small organic-carbon rich particles from bulk sediment (Millward et al. 2001). In the present study, ingestion of Cd associated with the daily meal and/or Cd associated with sediment particles provided a route of exposure for Cd bioaccumulation in addition to aqueous uptake. Because Phen had no affect on either Cd bioaccumulation in aqueous exposure or on dissolved Cd concentrations in sediment bioassays, the observed increase in Cd bioaccumulation when exposed to Cd-Phen mixtures in sediment likely resulted from an increase in Cd uptake via feeding selectivity or by processes associated with digestion.

The contribution to metal bioaccumulation from food has been linked to ingestion rate (IR), assimilation efficiency (AE), the concentration of metal in ingested food (C_i) and elimination rate of metal accumulated via feeding (k_{ef}) in a bioenergetic kinetic model (Wang and Fisher 1999). PAHs have been observed to increase Cd AE from contaminated food (Fair & Sick 1983), and to alter sediment-particle selectivity (Millward et al. 2001). Alterations in food selectivity caused by PAHs may therefore influence exposure and bioaccumulation of co-occurring contaminants, including metals. Additional experiments are required to quantify Phen effects on model parameters associated with the bioenergetic kinetic model for *H. azteca*. 
Because parameters characterizing metal uptake from ingestion may not be independent of one another, (e.g. Phen-mediated increases in more than one model parameter would result in multiplicative affects on bioaccumulation rate) the use of the bioenergetic model is necessary to understand how Phen affects Cd bioaccumulation.

**Conclusions**

The current basis for assessing ecotoxicological effects of contaminant mixtures in natural environments is based heavily on mixture toxicology with considerably less emphasis on environmental science and biology (Altenburger et al. 2004, Escher & Hermens 2002, Van Leeuwen et al. 1996). Understanding how contaminants interact toxicologically is important, but does not provide all of the information necessary for assessing what effects will occur in natural populations encountering contaminant mixtures in a diversity of natural environments. A major strength our understanding of ecotoxicology lies in understanding contaminant bioavailability and understanding how organisms are exposed to chemicals in natural environments. Without this knowledge, understanding toxicological mechanisms of combined contaminants does not help regulators predict and prevent adverse environmental effects. The current study demonstrated that the mixture toxicology of a metal and PAH in *Hyalella azteca* is likely to be independent. However, when organisms encountered the combined contaminants in sediment, lethality increased dramatically and appeared to result from exposure-mediated increases in metal bioaccumulation. These results demonstrate the importance of establishing a holistic view of contaminant mixture effects so that environmental quality can be accurately assessed and natural environments protected.

**LITERATURE CITED**


Escher BI, Hermens JLM (2002) Modes of action in ecotoxicology: Their role in body burdens, species sensitivity, QSARs, and mixture effects. Environ Sci Technol 36:4201-4217

Fair PH (1986) Interaction of benzo(a)pyrene and cadmium on GSH-S-transferase and benzo(a)pyrene hydroxylase in the black sea bass Centropristis striata. Arch Environ Contam Toxicol 15:257-263


Gust KA (in press) Joint toxicity of cadmium and phenanthrene in the freshwater amphipod Hyalella azteca. Arch Environ Contam Toxicol


Moreau CJ, Klerks PL, Haas CN (1999) Interaction between phenanthrene and zinc in their toxicity to the sheepshead minnow (Cyprinodon variegatus). Arch Environ Contam Toxicol 37:251-257


Viarengo A, Bettella E, Fabbri R, Burlando B, Lafaurie M (1997) Heavy metal inhibition of EROD activation in liver microsomes from the bass Dicentrarchus labrax exposed to organic xenobiotics: Role of GSH in the reduction of heavy metal effects. Mar Environ Research 44:1-11


CHAPTER 5

EXPOSURE TO CADMIUM-PHENANTHRENE MIXTURES ELICITS COMPLEX TOxico RESPONSES IN THE FRESHWATER TUBIFICID OLIGochaETE

ILYODRILUS TEMPLETONI
Environmental pollution often occurs as a mixture of various classes of chemical constituents (Kennicutt et al. 1996). The joint-toxicity of combined pollutants to organisms in natural environments can be complex and is related to the chemistries of the individual compounds, environment-specific bioavailability, bioaccumulation, toxicological modes of action and possible interactions among contaminants once bioaccumulated. Regulatory agencies often use risk assessment data based on single-contaminant toxicity tests to estimated maximum allowable concentrations of individual chemicals within a given environment. In areas where contaminant mixtures are present, environmental risk is typically determined assuming concentration-additive toxicity (USEPA 2000), which is defined as toxicity proportional to the summed toxicities of each individual contaminant concentration (Cassee et al., 1998; Broderius, 1991). Concentration-additive toxicity has been demonstrated to be an effective predictor of the overall toxicity of combined chemicals that have similar modes of toxic action and/or those having related quantitative structure-activity relationships (QSARs) (Van Leeuwen et al., 1996). Many organic contaminants fit this relationship well (Faust et al., 2000; Grimme et al., 1996; Broderius et al., 1995; and Broderius, 1991) and predictive models have been developed that successfully assess the joint toxicity of PAH mixtures (Di Toro and McGrath, 2000; Swartz et al., 1995).

Contaminants with dissimilar modes of toxic action, on the other hand, are generally hypothesized to elicit independent joint toxicity, which occurs when the contaminants in a mixture have no influence on one other’s toxic effects, thus resulting in less than concentration additive toxicity (Price et al., 2002; Faust et al., 2000; Cassee et al., 1998; Broderius, 1991). Because 95% of all toxicity studies investigate singular compounds (Yang 1994), the assumption
that concentration-additive or independent toxicity is representative of contaminant mixtures
(eespecially among dissimilar classes of contaminants) remains largely untested. Furthermore,
even if dissimilar chemicals elicit independent toxicological effects, contaminant mixtures may
elicit unexpected interactive effects facilitated via changes in environmental exposure. For
example, Gust (in press) observed that Cd-Phen mixtures elicited synergistic lethal effects in
*Hyalella azteca* in sediment exposures, but not in aqueous exposures. Further investigation has
suggested the toxicology between Cd and Phen is likely independent, but sediment-mediated
effects of this mixture alter bioaccumulation rates (Chapter 4). Additional investigations are
needed to test if and when the assumption of independence among dissimilar chemicals is robust
eough for use in ecological risk assessment protocols, especially in sediments.

Because metals and hydrocarbons are generally dissimilar in terms of their molecular
structure and modes of toxic action, they are hypothesized to elicit independent toxic effects. A
literature review of studies investigating metal-hydrocarbon mixtures (Chapter 2), however
suggests effects can be additive, independent, or interactive (synergistic or antagonistic).
Twenty six out of 31 studies suggest toxicity is interactive, with an overwhelming trend toward
synergism (22 out of 31, Chapter 2). This is of concern because mixtures of heavy metals and
PAHs may become prevalent in benthic and wetland sediments as a result of urbanization and
industrial contamination (Klerks 2001, 1999, Callender and Rice 2000, Van Metre et al. 2000,

We investigated the joint-toxicity of metal-hydrocarbon mixtures in sediments using the
polycyclic aromatic-hydrocarbon (PAH) phenanthrene (Phen) and the toxic heavy metal Cd as
model contaminants. Freshwater sediment toxicity bioassays were conducted with the bulk-
deposit feeding benthic tubificid oligochaete *Ilyodrilus templetoni* (Southern) where lethal and
sublethal (feeding rate, burrowing avoidance and tissue necrosis) endpoints were observed. The sublethal endpoints investigated were chosen to serve as relevant indicators of stress, and contributors to lethality. Alterations in feeding rate resulting from exposure to combined contamination may modify uptake of a given contaminant via the dietary route, which is an especially important route of uptake for bulk deposit feeders such as tubificids (Lu et al. 2003, Wang and Fisher 1999). Burrowing avoidance serves as an indicator of potentially toxic conditions in sediment and lessens exposure to contaminants associated with sediment and porewater. Tissue necrosis is also an indicator of toxic stress, and specialized tissue loss, such as autotomy, has been found to be a sensitive indicator of metal stress and a possible mechanism of metal depuration (Lucan-Bouché et al. 1999, 2000). The null hypothesis for the present study was: The joint-toxic effects of Cd and Phen in sediments is independent. The objective was to determine the joint-toxic effects of sediment-bound metals and PAHs in benthic organisms and, in broader scope, to test if the assumption of independent toxicity among dissimilar contaminants (metals and hydrocarbons) is robust enough for use in ecological risk assessment protocols for sediments.

**MATERIALS AND METHODS**

**Sediment Preparation**

Sediment used in all bioassays was collected from Bayou Manchac, a relatively uncontaminated freshwater bayou near Baton Rouge, Louisiana, USA. Sediment characteristics and methods for amending Cd and phenanthrene to sediments are described in Gust (in press). Amended sediments were stored at 4°C for no more than 7 d prior to bioassay initiation, and concentrations of Cd and Phen were measured in sediments collected at experiment initiation. Cadmium concentrations were measured by inductively-coupled argon plasma
spectrophotometry (ICP-AES) and Phen concentrations by high-performance liquid chromatography (HPLC). Sediment extraction methods and analytical procedures for contaminant analyses are described in Gust (in press).

**Laboratory Culture and Test Organisms**

*Ilyodrilus templetoni* (Southern) cultures were maintained at 23 ± 1°C in multiple 2-L round aquarium bowls containing sediment and overlying water. Culture sediments consisted of Better Homes and Gardens™ potting soil that passed through a 2 mm sieve. Culture chambers were individually aerated and experienced a natural light cycle of indirect light. Worms were fed mixed-grain baby cereal twice a week with supplemental spinach amendments once a month. Culture water and sediment were replaced every 3 weeks and 6 months respectively. Prior to initiation of toxicity bioassays, ten or twenty (depending on experiment) adult *I. templetoni* were transferred to Petri dishes that were randomly assigned to experimental treatments. The mean initial dry weight of adult *I. templetoni* used in toxicity bioassays was 0.36 ± 0.07 mg.

**Phenanthrene Effects on Feeding Rate**

Tubificid feeding rate is a sensitive indicator of sediment-hydrocarbon contamination (Lotufo and Fleeger 1996). *I. templetoni* feeds on the relatively limited organic carbon fraction adsorbed to sediment particles, thus requiring them to ingest several times their body weight of sediment per day to maintain a positive energy budget. Only a small fraction of the total organic carbon is assimilated, making egestion rate a close approximation of feeding rate.

The effect of Phen on *I. templetoni* feeding rate was tested in an experiment with a randomized design including 7 treatments, each including 6 replicates. Measured Phen concentrations in experimental treatments were: 0, 7, 51, 109, 112, 131, and 193 mg kg⁻¹ dry weight sediment. Treatment sediments in amounts that approximated a 50:1 TOC / worm dry
weight ratio (approximately 17 mL replicate\(^{-1}\)) were inserted into 50 mL glass centrifuge tubes (USEPA, 2000). Twenty-five mL of artificial pond water (APW, 0.5 mM NaCl, 0.05 mM KCl, 0.2 mM NaHCO\(_3\), 0.2 mM MgCl\(_2\), 0.4mM CaCl\(_2\)) was added to each replicate and allowed to equilibrate with sediment in the dark for 24 h. Feces collectors consisting of polyester filter floss topped with 2 layers of gauze were each held in place at the sediment-water interface with a polyvinyl chloride split ring (Lotufo and Fleeger 1996). APW was replaced 1 h prior to experiment initiation and replaced every 2 d thereafter. Twenty randomly selected adult worms were transferred into each replicate, and were observed to pass through the feces collector and burrow into the sediment shortly after insertion. Experimental treatments were incubated at 25 ± 1°C in the dark to minimize photo-oxidation of Phen.

Feces were collected every 2 d, oven dried at 70°C and then dry weights were recorded. On day 10, treatment sediments were washed onto a 125-µm sieve and the number of surviving worms was recorded. Surviving *I. templetoni* were transferred to Petri dishes containing APW and allowed to clear their guts for 12h. After gut clearance worms were transferred to pre-weighed 8 mL glass vials, frozen and later freeze dried. The average dry weight worm\(^{-1}\) was calculated for each replicate.

At the end of the 10-d feeding bioassay, mortality in each treatment was low (< 10%), and no treatment varied significantly from the control (P = 0.05). Day-10 egestion rate data were unreliable due to deterioration of the feces collectors and were excluded from subsequent analyses. Dry weight measurements for egested material were summed over the 8-d period, and the average egestion rate day\(^{-1}\) was calculated. Because feeding rate is approximately equal to egestion rate for *I. templetoni*, egestion rate will be referred to as feeding rate for the remainder of this paper. To account for the variability in worm size between replicates, feeding rate was
expressed in terms of worm dry weight for each replicate (g dry wt. egested material g dry wt. worm$^{-1}$ day$^{-1}$). The affect of Phen on feeding rate was tested using ANOVA, and the concentration-response relationship between sediment Phen concentration and feeding rate was developed using linear regression. Analyses were conducted using SigmaStat 2.01 (Jandel Scientific).

A separate experiment was conducted to test the effect of sediment-amended Cd on *I. templetoni* feeding rate. The experimental design and methods used to conduct this bioassay are equivalent to those described above. Treatments included 11 Cd-spiked sediments and an unamended control, each having 6 replicates. Measured Cd concentrations in treatments included: 0, 10, 40, 88, 124, 165, 520, 803, 833, 1183, 1346 and 1396 mg kg$^{-1}$ dry weight sediment. At the end of the 10-d bioassay, mortality in each treatment was low (< 10%), and no treatment varied significantly from the control (P = 0.05). For consistency, the feeding-rate data for d-10 were excluded and the average dry weight of egested material d$^{-1}$ was quantified. The affect of Cd on feeding rate was tested using ANOVA, and the concentration-response relationship between sediment Cd concentration and feeding rate was developed using linear regression.

An experiment with a randomized design and 4 x 3 factorial treatment arrangement was conducted to test the effects of Cd-Phen mixtures on *I. templetoni* feeding rate. The interaction term was used as a test for toxic interactions between contaminants. Four Phen treatments 0, 50, 250, and 500 mg kg$^{-1}$ dry weight sediment (Nominal, see Table 5.1 for measured concentrations) were combined with 3 Cd treatments: 0, 497, and 887 mg kg$^{-1}$ dry weight sediment (measured concentrations). No significant mortality was detected compared to controls. Treatment effects on average feeding rate d$^{-1}$ for the 8-d period were analyzed by analysis of covariance
(ANCOVA) on natural-log-transformed data (due to non-homogeneity of variance) using SAS® software (Release 8.2, SAS Institute Inc.).

Table 5.1. Measured sediment phenanthrene concentrations from experiment testing Cd-phenanthrene mixture affects on *Ilyodrilus templetoni* feeding rate. All contaminant concentrations in mg kg⁻¹. Values represent mean (n = 2).

<table>
<thead>
<tr>
<th>Nominal Phenanthrene Concentration</th>
<th>0 Cd Conc. (0)*</th>
<th>500 Cd Conc. (497)*</th>
<th>1000 Cd Conc. (887)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>37</td>
<td>47</td>
<td>36</td>
</tr>
<tr>
<td>250</td>
<td>188</td>
<td>161</td>
<td>191</td>
</tr>
<tr>
<td>500</td>
<td>318</td>
<td>314</td>
<td>370</td>
</tr>
</tbody>
</table>

* Measured Cd Concentrations

**Effect of Cd-Phenanthrene Mixtures on Mortality**

Phenanthrene caused no detectible mortality compared to controls at concentrations approaching sediment saturation (~450 mg kg⁻¹) based on sediment-organic-carbon and equilibrium partitioning theory. A randomized design with a 4 x 8 factorial treatment arrangement was used to test the effect of sublethal concentrations of Phen on Cd toxicity. Phenanthrene treatments included: 0, 81, 189, 378 mg kg⁻¹ dry weight sediment (measured concentrations) and 8 Cd concentrations 1000, 1300, 1600, 1900, 2200, 2500, 2800, 3100 mg kg⁻¹ dry weight sediment (see Table 5.2 for measured concentrations). Each treatment included 4 replicates containing 10 randomly selected worms replicate⁻¹. Sigmoid concentration-response curves were generated, and LC₅₀ values with 95% confidence intervals (C.I.) were calculated using probit analysis. Non-overlapping 95% C.I.s were used as criteria for identifying significant differences in mortality among concentration-response curves.
Table 5.2. Measured sediment Cd concentrations from Cd-Phenanthrene mixture toxicity bioassay. All contaminant concentrations measured as mg kg\(^{-1}\). Values represent means (n = 2).

<table>
<thead>
<tr>
<th>Measured Phenanthrene Conc. (mg kg(^{-1}))</th>
<th>Measured Cd concentration (mg kg(^{-1})) within each treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1  2  3  4  5  6  7  8</td>
</tr>
<tr>
<td>81</td>
<td>853 1323 1651 1103 2100 2108 2392 2524</td>
</tr>
<tr>
<td>189</td>
<td>951 1357 1547 1545 1866 2328 2576 2885</td>
</tr>
<tr>
<td>378</td>
<td>959 1307 1569 1562 2011 2257 2462 2741</td>
</tr>
</tbody>
</table>

**Effect of Cd-Phenanthrene Mixtures on Burrowing Avoidance**

During the lethality experiment described above, chambers were examined daily for burrowing avoidance. Worms were considered to be avoiding sediment when 2/3 or more of their total body length was extended out of the sediment. Worms that avoided sediment were enumerated in each replicate and the percentage burrowing avoidance was calculated. These data were averaged over the 10-d bioassay for each treatment to determine the mean burrowing avoidance. High mortality (50-100%) occurred within 24 h of experiment initiation in the upper range of Cd treatments (target concentrations of 2500 – 3100 mg kg\(^{-1}\); see Table 5.2 for measured concentrations). For this reason, burrowing avoidance for the entire range of combined Cd and phenanthrene treatments was only available for the initial observations at 2.5 h. ANCOVA was used to test for the effects of increasing Cd and Phen concentrations and their interaction for initial burrowing avoidance. Data were square-root transformed in order to meet the assumptions of ANCOVA.

The average burrowing avoidance over the 10-day bioassay was calculated for treatments that had > 50% survival by the end of the experiment. In order to maintain an orthogonal data
set for investigation of interactions, the size of the statistical model was also limited by the
treatment with the lowest survivorship. In this case, only the two lowest Cd concentrations could
be compared with each corresponding Phen concentration (2 x 4 factorial treatment
arrangement). A conservative estimate of percentage burrowing avoidance for each replicate
was calculated by dividing the number of worms found avoiding sediment by the number of
worms surviving at the end of the test or by dividing by the highest number of worms found
avoiding sediment prior to d-10. ANCOVA was used to analyze square-root transformed data.

Effect of Cd-Phenanthrene Mixtures on Anterior Tissue Necrosis

During the daily observations of the test chambers, tissue necrosis in the anterior end of
worms was observed. The condition was quantified as the percentage of surviving worms
exhibiting “anterior necrosis” at experiment end. Due to complete mortality in the upper range
of Cd concentrations, the number of treatments was reduced to provide an orthogonal data set for
statistical analysis. A 3 x 4 factorial treatment arrangement including the 3 lowest Cd
treatments concentrations (Table 5.2) and all four levels of Phen were analyzed by ANCOVA on
square-root transformed data.

RESULTS

Individual Contaminant Effects

Phenanthrene significantly reduced *Ilyodrilus templetoni* feeding rate (Figure 5.1A, P <
0.001). Dunnet’s multiple comparisons test (P = 0.05) indicated significant reductions in feeding
rate compared to the control at Phen concentrations at and above 112 mg kg⁻¹. Data yielded the
regression [(feeding rate) = 7.977 – (0.0264 * Phen concentration), P < 0.001, R² = 0.366], which
was used to compare the individual effects of Phen and Cd on *I. templetoni* feeding rate (next
section).
Cadmium significantly reduced *I. templetoni* feeding rate (Figure 5.1B, \(P < 0.001\)). With the exception of the 124 mg kg\(^{-1}\) Cd treatment, none of the “low” Cd treatments (10, 40, 88, 165, 520 mg kg\(^{-1}\)) caused significant reductions in feeding rate compared to the control as indicated by Dunnet’s multiple comparisons test (\(P = 0.05\)). In all other Cd treatments, worms were observed to have significantly lower feeding rates than the control. As the concentration of Cd increased, feeding rate decreased \([\text{feeding rate} = 8.209 – (0.00331 \times \text{Cd concentration}), P < 0.001, R^2 = 0.473; \text{Figure 5.1B}]\).

**Cd-Phenanthrene Mixture Effects**

The concentration-responses for Cd-Phen mixture effects on feeding rate did not differ significantly from the concentration-response generated for Phen alone (Figure 5.2A). Results of ANCOVA indicated that Phen significantly reduced *I. templetoni* feeding rate (\(P < 0.0001\)) and Cd did not (\(P = 0.5561\)). No significant interaction occurred among contaminant treatments (\(P = 0.1625\)) suggesting the joint effects of Cd and Phen on feeding were independent in *I. templetoni*.

Range-finding experiments indicated Phen caused no detectable lethality even when loaded to sediment saturation, (\(\sim 450\) mg kg\(^{-1}\)). Cadmium elicited mortality in *I. templetoni* at relatively high concentrations (> 1300 mg kg\(^{-1}\)). *I. templetoni* consistently demonstrated a threshold lethal response when exposed to Cd alone or in combination with Phen in 10-d bioassays (Figure 5.3). Within relatively small increments of increasing Cd concentrations (< 200 mg kg\(^{-1}\)), mortality shifted from equivalence with uncontaminated controls to complete mortality.

The combination of Cd and Phen at all Phen concentrations (81, 189 and 378 mg kg\(^{-1}\)) significantly increased survivorship compared to worms exposed to Cd alone (Figure 5.3). The LC\(_{50}\) values for Cd alone and Cd in combination with 81, 189 and 378 mg kg\(^{-1}\) Phen were 1375
Figure 5.1. Effect of phenanthrene (A) and Cd (B) on *Ilyodrilus templetoni* sediment feeding rate. Symbols represent means ±1 S.D. (n = 6). Phenanthrene and Cd concentrations expressed in mg kg$^{-1}$ dry weight sediment.
(1340-1412), 1929 (1852-2005), 1770 (1701-1840), 1779 (1682-1883) mg kg⁻¹ Cd respectively (95% C.I.s in parentheses). Phenanthrene alone caused no lethal effects, and based on the assumptions of either independent or concentration-additive toxicity, should have had no effect on the concentration-response relationship of Cd. The greatest enhancement in survivorship, a 40.1% increase in Cd LC₅₀, occurred at the lowest Phen concentration, suggesting that optimal survivorship in the presence of lethal concentrations of Cd may occur at low Phen concentrations.
Figure 5.3. Effect of Cd-phenanthrene mixtures on *Ilyodrilus templetoni* lethality in a 10-d sediment bioassay. Symbols represent treatment means and error bars represent standard error (n = 4). Letters (A-D) identify each concentration-response curve, and stars (*, **, ***) denote statistically significant differences between concentration response curves. Phenanthrene and Cd concentrations are displayed in mg kg\(^{-1}\) dry wt. sediment. Phen = phenanthrene.

**Burrowing Avoidance**

Results of ANCOVA suggested a significant effect of Phen (P < 0.0001) and Cd (P = 0.0196) on burrowing avoidance at 2.5 h of exposure, and a significant interaction was found between these parameters (P = 0.0005). Tukey’s test (P = 0.05) indicated a significant increase in burrowing avoidance at the highest Phen concentration (378 mg kg\(^{-1}\)) where avoidance ranged
from 53-80% (Figure 5.4A). Limited burrowing avoidance occurred in treatments containing Cd-alone and Cd with co-occurring low and moderate Phen concentrations (81 and 189 mg kg\(^{-1}\)). The significant interaction term was likely driven by the increase in avoidance observed at the highest Cd concentrations.

Statistical analysis of the average burrowing avoidance over 10 d revealed significant effects of Phen (P < 0.0001), Cd (P = 0.0194) and their interaction (P < 0.0001). Patterns of avoidance are shown in Figure 5.4B. Tukey multiple comparisons test (P = 0.05) indicated significant differences among all Phen concentrations. Burrowing avoidance did not increase with increasing Cd concentration when Phen was present, however, burrowing avoidance increased dramatically from 5% at 934 mg kg\(^{-1}\) to nearly 60% at 1305 mg kg\(^{-1}\) when \(I.\) templetoni was exposed to Cd alone (Fig 4B). The significant interaction term was most likely driven by this occurrence.

**Anterior Tissue Necrosis**

The occurrence of anterior necrosis was the most pronounced in the Cd alone treatment (Figure 5.5). Results of ANCOVA indicated significant effects of Cd (P < 0.0001) and that a significant Cd-Phen interaction occurred (P = 0.0266). Although the Phen main effect was not significant (P = 0.3372), Tukey’s multiple comparisons (P = 0.05) indicated a significant increase in tissue necrosis when \(I.\) templetoni was exposed to Cd alone compared to worms exposed to the Cd-Phen mixture (Figure 5.5). It should be noted that data for the highest Cd alone treatment consisted of the average of all replicates with only one surviving worm in each. However, the incidence of tissue necrosis was noted in 100% of worms in each test chamber during earlier observations before lethality reached 50%, suggesting these data are meaningful. Nearly all worms in treatments where 100% mortality was reached prior to the end of the assay showed signs of anterior necrosis prior to death.
Figure 5.4. Percent burrowing avoidance of *Ilyodrilus templetoni* versus Cd concentration in combination with phenanthrene (A) at 2.5 h exposure and (B) averaged over the 10 day bioassay. Symbols represent means ±1 S.D. (n = 4). Cd and phenanthrene concentrations in mg kg\(^{-1}\) dry wt. sediment. Phen = phenanthrene.
Figure 5.5. Percent incidence of anterior necrosis in *Ilyodrilus templetoni* versus Cd concentration in combination with phenanthrene at the end of the 10-d bioassay. Symbols represent means ±1 S.D. (n = 4). Cd and phenanthrene concentrations in mg kg$^{-1}$ dry wt. sediment. Phen = phenanthrene.

**DISCUSSION**

The observed tolerance of *I. templetoni* to high concentrations of sediment-associated Cd (in excess of 1300 mg kg$^{-1}$) is likely the result of a relatively high species-specific metal tolerance (Gust unpublished) and limited bioavailability of Cd associated with the muddy sediment used in our bioassays. *I. templetoni* exhibits lethality in aqueous exposures at Cd concentrations comparable to related freshwater oligochaetes including *Lumbriculus variegatus* and *Tubifex tubifex* (Rathore and Khangarot 2003, USEPA 2000), which are relatively metal-
tolerant (Milani et al. 2003). The 24-h LC$_{50}$ for *I. templetoni* exposed to aqueous Cd was determined to be 2.4 mg L$^{-1}$ (95% C.I. 2.1–2.7) in ‘reconstituted water’ (hardness 90-100 mg L$^{-1}$ as CaCO$_3$ at pH 7.8-8.2) (Gust unpublished). Extreme tolerance to Cd (low mortality at 34,000 mg kg$^{-1}$ total Cd) has also been observed in natural populations of tubificid oligochaetes living in heavily Cd-polluted sediments (Wallace et al. 1998, Klerks and Bartholomew 1991, Klerks and Levinton 1989). However, the presence of Phen reduced the lethal toxicity of Cd in *I. templetoni* increasing the LC$_{50}$ of Cd by as much as 40.1% (95% C.I. 38.2-42.0%; Figure 5.3). Phenanthrene alone caused no detectible lethality even when loaded to sediment saturation. Therefore, the lethal joint toxicity of Cd and Phen in this sediment bioassay is characterized as antagonistic for *I. templetoni* (Cassee et al. 1998). There are several possible mechanisms that may have contributed to the antagonism and each are discussed below.

Bioaccumulation of metals by deposit feeders occurs predominantly via uptake from feeding on contaminated sediments (Wang and Fisher 1999). Therefore, the rate of sediment ingestion likely influenced Cd bioaccumulation in our experiments. Cadmium and Phen each reduced feeding rates in *I. templetoni* with increasing concentration (Figure 5.1). Feeding rate proved to be a sensitive indicator of Phen contamination, with significant reductions compared to controls detected at concentrations as low as 112 mg kg$^{-1}$ Phen (Fig. 1A). *Limnodrilus hoffmeisteri* also demonstrated feeding-rate sensitivity to sediment containing Phen (Lotufo and Fleeger 1996). Feeding rate was a less sensitive indicator of sediment-Cd contamination (Fig. 1B). Similarly, *Capitella* sp. I feeding rate was unaffected when exposed to sediment-associated Cd even though their Cd body burden increased dramatically during feeding (Selck et al. 1998). The combination of Cd and Phen elicited an independent joint toxic response for feeding rate in *I. templetoni*. Reduced feeding rates appeared to be driven primarily by the concentration of Phen in the sediment (Figure 5.2).
Bioaccumulation kinetics models, such as the bioenergetic-based kinetic model proposed by Wang and Fisher (1999), utilize physiological parameters including metal uptake from the dissolved phase ($k_u$), assimilation efficiency (AE) of metal from ingested material, ingestion rate (IR) and metal efflux rates ($k_e$) to predict animal body burdens and thus exposure. We estimated these model parameters for *I. templetoni* under our experimental conditions (Chapter 6). Assuming that each parameter value, except for IR, remains constant in the presence of Phen and assuming that a high percentage of the Cd body burden accumulates through the ingestion route of uptake (Wang and Fisher 1999), Phen-induced reductions in IR are predicted by model calculations to reduce Cd body burdens in *I. templetoni* by as much as 72%. This large impact suggests that Phen may confer increased survivorship by lowering Cd bioaccumulation rates.

Aside from feeding-rate effects, it is possible that Phen may alter other parameters in the kinetic model that contribute to Cd bioaccumulation. Moreau et al. (1999) indicated that Phen has the capacity to reduce metal bioaccumulation from the dissolved phase ($k_u$). In that study Phen reduced Zn bioaccumulation in sheepshead minnow (*Cyprinodon variegatus*), which was associated with decreased lethality. As well, Phen-induced modifications in Cd AE, $k_u$, and $k_e$ could also contribute to reductions in Cd bioaccumulation. Preliminary results from kinetic-uptake bioassays with *I. templetoni* suggest tissue-Cd concentrations are reduced in the presence of Phen (Chapter 6).

Phenanthrene may also alter Cd toxicity by modifying Cd bioavailability in sediments or by influences over the behavior, physiology or toxicology of *I. templetoni*. For example, if Phen modified the physico-chemistry of sediments in a way that enhanced Cd partitioning to recalcitrant environmental ligands (such as Cd binding to acid volatile sulfides), Cd bioaccumulation would likely be reduced. The potential for hydrocarbon contamination to alter
metal bioavailability remains to be tested, and is of critical importance in natural environments where microbial communities control sediment biogeochemistry.

Sediment-burrowing avoidance was a predominant behavioral response in *I. templetoni* exposed to the highest Phen concentrations which could potentially reduce exposure to Cd. Burrowing avoidance is often considered a protective mechanism for organisms, providing escape from the toxic chemicals associated with polluted sediments. Burrowing avoidance was a strong indicator of high levels of Phen contamination, while medium and low concentrations of Phen elicited minimal avoidance. Burrowing avoidance in response to Phen has also been noted in the freshwater oligochaete *Limnodrilus hoffmeisteri* at moderate to high sediment concentrations (143-612 mg kg$^{-1}$, Lotufo and Fleeger 1996) and at low concentrations (at and above 56 mg kg$^{-1}$) in the estuarine copepod *Schizopera knabeni* (Lotufo 1997). In the present study, Cd alone caused little burrowing avoidance up to the threshold of mortality. The response pattern of *I. templetoni* to the combined pollutants was dependent on Phen concentration (Figure 5.4). Although avoidance provides a potential means for reducing exposure to sediment-bound Cd, it did not enhance survivorship compared to *I. templetoni* exposed to Cd combined with low to moderated Phen concentrations (Figure 5.3).

The presence of Phen reduced Cd-mediated tissue necrosis in *I. templetoni* providing additional evidence of antagonism. The loss of tissue resulting from exposure to toxic chemicals may come through the death of tissues (necrosis), or via the discarding of appendages damaged by toxic insult (autotomy). Caudal tissue autotomy was elicited by heavy metals such as Cd, Cu, and Pb in oligochaete worms including *Lumbriculus variegatus* and *Tubifex tubifex* (Lucan-Bouche et al. 1999, 2000, 2003) and has also been considered a means of metal depuration (Lucan-Bouche et al. 1999). Tissue loss observed in the present study was not characteristic of
autotomy in oligochaete worms (Lesiuk and Drewes 1999). In contrast, tissue necrosis occurred in the anterior end of *I. templetoni*, and the dead tissues remained associated with the worm until decomposed. Tissue necrosis has been reported in *Tubifex tubifex* in response to Cd exposure (Gillis et al. 2002). A reduction in necrosis was observed in Cd-Phen mixtures similar in pattern to that observed for the lethal response. Presumably, the reduction in frequency of anterior necrosis was directly related to the mechanisms contributing to antagonistic lethality.

Finally, Phen may be involved in a true antagonistic toxicological interaction with Cd. For example, Phen-mediated interference with mechanisms that confer Cd toxicity and/or enhancement of Cd detoxification mechanisms could theoretically mitigate Cd toxicity. In the European eel *Anguilla anguilla*, Cd-PAH mixtures elicited greater production of both metal- and PAH-detoxification enzymes than expected by summing responses elicited by the individual chemicals (Lemaire-Gony and Lemaire 1992).

The 10 d bioassays conducted in the present study tested acute effects of Cd-Phen mixtures. Effects of chronic exposure to Cd-Phen mixtures have not been examined, but should be considered. Phenanthrene- and Cd-mediated reductions in *I. templetoni* feeding rate were associated with reductions in growth rates in the present bioassays (data not presented). Growth rates were negative (-2.59% growth day\(^{-1}\)) at high Phen concentrations compared to 0.35\% growth day\(^{-1}\) in control animals and -0.75% growth d\(^{-1}\) in worms exposed to low Phen concentrations. Reduced feeding as the result of chronic exposure to high Phen and Cd concentrations may impart additional lethal effects which were not detected in the acute bioassays presented here. The antagonistic effect of Phen was not linearly related to reductions in feeding rate (i.e., the lowest concentration of Phen reduced feeding rate slightly but conferred the maximum enhancement of survivorship when exposed to Cd). Effects of low Phen...
concentrations on feeding rate and growth were relatively minor, suggesting chronic effects of Phen at low concentrations may be negligible. If so, the antagonistic effect of Phen on Cd lethality may be long-lived.

To the best of our knowledge, no other studies have shown that a co-contaminant may alter the exposure of another contaminant. Our results suggest Phen-induced reductions in feeding rate can reduce exposure to sediment-bound Cd in bulk deposit feeders. Several toxicants have been shown to reduce feeding rate in a broad range of benthic taxa (Forrow and Maltby 2000, Blockwell et al. 1998, Lotufo and Fleeger 1996, Weston 1990). Given that sediments are frequently contaminated with multiple chemicals, it is plausible that feeding rate changes driven by one contaminant that alter a deposit-feeding organism’s exposure to co-contaminants may be a common occurrence. This type of relationship should be considered when attempting to predict the toxicity of chemical mixtures in natural environments.

**Implications and Importance**

The concepts of additive and independent mixture toxicology are founded on the fundamental toxicological unit, the dose. In environmental exposures, especially in sediments, determining the expected dose may be complex. In the present study, a PAH reduced exposure to a heavy metal, thereby likely reducing the heavy metal dose. Conversely, the same exposure regime elicited synergistic lethal effects in *H. azteca* (Gust in press) in which PAH increased the heavy metal dose (Chapter 4). These experiments indicate that exposure source may be more important than toxicological interactions in determining contaminant mixture effects in sediment environments. There are 2 fundamental challenges to expanding the concepts of dose-additive and independent toxicity to ecological risk assessment. (1) Although, many “similar” chemicals are well characterized by dose-addition, the assumption of independent joint toxicology for dissimilar chemicals should be used with caution. Most combinations have never been tested.
(2) Observed interactions may be manifested via mechanisms unrelated to mixture toxicology. Our studies indicate that contaminant mixtures in complex exposure sources (i.e. sediments) may elicit unexpected effects on exposure and subsequent toxicity. If this trend is widespread, understanding how species are exposed, determining route of uptake and understanding how environmental characteristics affect exposure may be more important in determining mixture effects than knowing mixture toxicology. Based on these challenges, we cannot condone the use of dose-additive and independent toxicology as a basis for ecological risk assessment.

**LITERATURE CITED**


Gust KA (In press) Joint toxicity of cadmium and phenanthrene in the freshwater amphipod Hyalella azteca. Arch Environ Contam Toxicol


Klerks PL, Moreau CJ (2001) Heritability of resistance to individual contaminants and to contaminant mixtures in the sheepshead minnow (Cyprinodon variegatus). Environ Toxicol Chemi 20:1746-1751


Lotufo GR (1997) Toxicity of sediment-associated PAHs to an estuarine copepod: effects on survival, feeding, reproduction and behavior. Mar Environ Research 44:149-166


Lucan-Bouche ML, Biagianti-Risbourg S, Arsac F, Vernet G (1999) An original decontamination process developed by the aquatic oligochaete Tubifex tubifex exposed to copper and lead. Aquat Toxicol 45:9-17


Moreau CJ, Klerks PL, Haas CN (1999) Interaction between phenanthrene and zinc in their toxicity to the sheepshead minnow (Cyprinodon variegatus). Arch Environ Contam Toxicol 37:251-257


U.S. Environmental Protection Agency (2000b) Supplementary guidance for conducting health risk assessment of chemical mixtures. Washington DC, Office of Research and Development EPA/630/R-00/002


Weston DP (1990) Hydrocarbon bioaccumulation from contaminated sediment by the deposit-feeding polychaete Abarenicola pacifica. Mar Biol 107:159-169

INTRODUCTION

Although interactive toxicity between metals and hydrocarbons has been documented (Gust in press, Millward et al. 2004, van den Hurk et al. 1998, Viarengo et al. 1997, Forlin et al. 1986) the mechanisms underlying interactive effects in environmentally relevant exposures have been sparsely investigated. In chapter 5 antagonistic lethal effects were observed in *Ilyodrilus templetoni* when sublethal concentrations of phenanthrene (Phen) were combined with Cd in sediment exposures. The purpose of the present study was to determine the mechanisms responsible for the observed antagonism.

Alterations in contaminant bioavailability resulting from interactions between contaminants in complex mixtures have been observed. For example, diesel contamination in microcosm experiments has been observed to increase metal retention in sediments (Millward et al. 2004). In the present study, tests of Phen effects on Cd bioavailability were conducted using simultaneously extractable metal - acid volatile sulfide (SEM - AVS) relationships (Di Toro et al. 1992) combined with measurements of Cd concentrations dissolved in porewater and overlying water. The free ion state of cadmium (Cd$^{+2}$) is considered the most bioavailable fraction of metals in the environment (Di Toro et al. 2001, Chapman et al. 1998). AVS concentrations control the concentration of SEM that is free to partition into the highly bioavailable dissolved phase. Therefore, effects of Phen on AVS, SEM or dissolved Cd concentrations in porewater / overlying water have the potential to alter Cd bioaccumulation.

In aquatic organisms, metal bioaccumulation rates strongly influence metal toxicity (Barron et al 2002). The effects of Phen on Cd bioaccumulation and elimination rate kinetics were investigated in both sediment and water-only exposures. Any observed reductions in Cd bioaccumulation rates that are attributable to Phen effects may explain the antagonistic toxicity...
observed in worms exposed to the contaminant mixture. A bioenergetic-based kinetic model (Wang and Fisher 1999) was utilized to determine how Phen influenced Cd bioaccumulation. Experiments were conducted to determine all parameters associated with the kinetic model for *I. templetoni* exposed to Cd alone and Cd-Phen mixtures. The resulting parameter values were used to generate predicted Cd bioaccumulation kinetics curves that were compared with empirical bioaccumulation kinetics data. Also, affects of Cd on Phen bioaccumulation and biota sediment accumulation factor (BSAF) were tested in consideration of the contribution of Phen to toxicity. Results of the current study indicate that interactive toxicity among contaminants in chemical mixtures may occur via mechanisms unrelated to interactive toxicology. As an implication of these results, it is possible that constituents within contaminant mixtures may alter an organism’s exposure to co-occurring contaminants, thereby altering bioaccumulation and toxicity.

**MATERIALS AND METHODS**

**Sediment Preparation**

Sediment used in all bioassays was collected from Bayou Manchac, a relatively uncontaminated freshwater bayou near Baton Rouge, Louisiana, USA. Sediment characteristics and methods for amending Cd and phenanthrene (Phen) to sediments are described in Gust (in press). Concentrations of both Cd and Phen were measured in sediments at experiment initiation. Cadmium concentrations were measured by inductively-coupled argon plasma spectrophotometry (ICP-AES) and Phen concentrations by high-performance liquid chromatography (HPLC). Sediment extraction methods and analytical procedures for contaminant analysis are also described in Gust (in press). All Cd and Phen values listed below are measured concentrations taken at experiment initiation.
**Laboratory Culture**

*Ilyodrilus templetoni* (Southern) is a freshwater tubificid oligochaete that feeds by bulk deposit-feeding on benthic sediments. Culturing techniques used to rear *I. templetoni* are described in Chapter 5. Prior to initiation of toxicity bioassays, groups of ten or twenty (depending on experiment) adult *I. templetoni* were randomly assigned to experimental treatments. The mean initial dry weight of adult *I. templetoni* used in bioassays was 0.36 ± 0.07 mg.

**Effect of Phen on Cd SEM - AVS and Mortality**

The effects of Phen on both acid volatile sulfide (AVS) and simultaneously extractable metal (SEM) concentrations in sediments were tested. The relationship between SEM - AVS and mortality was also investigated to determine if reduced Cd lethality was a function of reduced metal bioavailability. Twenty randomly selected worms were exposed to sediments containing 0, 152 and 368 mg kg⁻¹ Phen combined with 5 concentrations of Cd ranging from 8.90-19.93 µmol g⁻¹ (see Table 6.1 for measured values) in 4 replicates. After a 10 d exposure period, overlying water was decanted from exposure vessels and approximately 3 mL of treatment sediment from 2 randomly selected replicates per treatment were each transferred to acid washed 20 mL glass vials. Oxygen was evacuated from sample vials immediately after insertion of sediment using a high-purity nitrogen gas stream. Sediment samples were sealed, stored in the dark at 4°C and analyzed for AVS and SEM following (Di Toro et. al. 1992) within 2 wk of sampling. After sediment samples were taken, worms were recovered from each replicate and, enumerated for survivorship. Effects of Phen on Cd lethality, and AVS concentration were tested using 2-way analysis of covariance (ANCOVA).
Table 6.1. Effects of phenanthrene (Phen) on AVS concentration, SEM for Cd and Cd-induced lethality in *I. templetoni*.

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<th>Cd SEM (µmol g(^{-1}))</th>
<th>AVS (µmol g(^{-1}))</th>
<th>SEM - AVS</th>
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**Effect of Phen on Cd Bioaccumulation and Elimination Kinetics in Water-Only Exposures**

A bioassay with randomized design and 2 x 3 x 6 factorial treatment arrangement was used to test effects of Phen on Cd bioaccumulation kinetics in water-only exposures. Contaminant treatments included 2 Cd concentrations (130 and 271 µg L\(^{-1}\)) combined with 0, 166 and 431 µg L\(^{-1}\) Phen. Ten randomly selected worms were exposed to 20mL of contaminant treatment solution in 50mL glass centrifuge tubes each replicated 4 times. Contaminant solutions were made in artificial pond water (APW, 0.5 mM NaCl, 0.05 mM KCl, 0.2 mM NaHCO\(_3\), 0.2 mM MgCl\(_2\), 0.4mM CaCl\(_2\)) to which 64 µCi of \(^{109}\)Cd radiotracer (PerkinElmer Life Sciences, Inc. Boston, MA, USA) was amended. Cadmium target concentrations were achieved by adding CdCl\(_2\) (98% purity, Sigma Chemical Co. Saint Louis, MO, USA) to treatment
solutions. Phen required dissolution in acetone before being amended to treatment solutions, therefore an aliquot of the acetone carrier was also amended to the 0 µg L⁻¹ Phen treatment. Tissue-Cd concentrations were measured after 0.5, 1, 2, 6, 12, and 24 h of exposure to contaminant treatments. At each time period, surviving worms were enumerated, rinsed thoroughly in deionized water 3x and transferred to gamma counting vials. After gamma-counting using a Perkin Elmer / Wallac Wizard 1470 gamma counter, worms were transferred to aluminum pans, oven dried at 70°C and then tissue dry weights were determined.

Samples collected for Cd analysis were acidified using trace metal grade HNO₃ immediately after collection, stored at 4°C and analyzed within 2 wk of experiment initiation. Phenanthrene samples were stored in the dark at 4°C and analyzed by HPLC within 2 d of experiment initiation. The activity of radiotracer in solution and in worm tissue was measured, and counts per minute (cpm) data were corrected for radioactive decay and for gamma-counting efficiency to calculate disintegrations per minute (dpm). The ratio of measured Cd concentration in treatment solution to total activity (dpm L⁻¹) was used to determine the dry-weight concentration of Cd found in I. templetoni tissues. The relationship between Cd concentration in solution and time of exposure on bioaccumulation was determined for each phenanthrene treatment using linear regression. Regression equations were used to calculate Cd uptake rate constants (k_u) for each treatment. The effects of Phen concentration, Cd concentration, time and their interactions on Cd bioaccumulation were tested by 3-way ANOVA using SAS® software (Release 8.2, SAS Institute Inc., Cary, NC, USA). Dunnett’s test was used compare Cd bioaccumulation in treatments containing Phen versus the Cd alone treatment. Data were natural-log transformed to meet the assumptions of ANOVA.

A group of worms undergoing the same exposure regime described above were exposed for 12 h, assayed for radioactivity and then transferred to 80 mL of clean APW in 100 mL plastic
beakers to allow Cd elimination. Within each replicate, worms were assayed for radioactivity after 1, 2, 6, 24, and 48 h elimination periods. Tissue-Cd concentrations were determined as described above. The relationship between Cd elimination and time for each Phen treatment was determined by linear regression. Cadmium elimination rate constants ($k_{ew}$) were calculated for each Phen treatment using equations derived from linear regression. Effects of Phen concentration, Cd concentration, time and their interactions were tested by 3-way ANOVA on repeated measures.

**Effect of Phen on Cd Assimilation Efficiency**

Experiments were conducted to test the effect of Phen on the assimilation efficiency (AE) of Cd from sediments in “novel” (unexposed) worms and worms pre-exposed to Cd. In pre-exposures, 2 groups of randomly selected worms were exposed for 48 h to either clean sediment or sediment containing 335 mg kg$^{-1}$ (measured concentration) of non-radioactive Cd. Groups of 100 worms were pre-exposed to 300 mL of treatment sediments in 1L beakers incubated at 23ºC in the dark. Pre-exposure vessels contained 600 mL of overlying water that was continuously aerated. After pre-exposure, worms were collected from treatment sediments following methods described in Chapter 5, transferred to 100mL plastic beakers and allowed to clear gut contents for 6 h. After gut clearance, worms were assigned to experimental treatments.

Pulse-chase exposure techniques (Wang and Fisher 1999a) were used to assess Cd assimilation efficiency (AE). Twenty randomly selected worms were exposed in 50 mL glass centrifuge tubes containing 15 mL of treatment sediment and 25 mL of overlying APW in 4 replicates. Treatment sediments contained 335 mg kg$^{-1}$ Cd (measured concentration) including 37 µCi of $^{109}$Cd radiotracer combined with 0 and 28 mg kg$^{-1}$ Phen. At experiment initiation, worms were introduced into exposure vessels and allowed to burrow and deposit-feed on
treatment sediments for 1 gut-passage time (40 min). After exposure, worms from each replicate were recovered from sediment, washed on a 125µm sieve and surviving worms were enumerated. Survivors were thoroughly rinsed in dechlorinated tap water, transferred to gamma-count vials and assayed for radioactivity. Worms were then transferred to 100mL plastic beakers containing clean APW and allowed to depurate gut contents. Each group of 20 worms was assayed for radioactivity after 3, 6 and 24 h of depuration.

A plot of Cd concentrations associated with worms tissue versus time was used to visually separate the effects of gut clearance and physiological elimination of Cd from tissues. Plots indicated that worms had completely depurated ingested sediments within 6h. Complete gut clearance has also been observed to occur within 6 h in the closely related tubificid oligochaete *Lumbriculus variegatus* (Mount et al.1999). Assimilation efficiency represents the percentage of a substance incorporated into tissues from food (Penry 1998). In order to correct for Cd lost by physiological elimination during gut clearance, linear regressions on data collected after completion of gut clearance were plotted for each treatment and y-intercept values were used to calculate initial tissue-Cd concentrations (Wang and Fisher 1999a). Initial tissue-Cd concentrations were then divided by the bulk-Cd concentration associated with worms prior to depuration, yielding AE. The effects of Phen, Cd pre-exposure and their combination on Cd AE were tested with 2-way ANOVA on natural log-transformed data (to meet assumption of homogeneous variance).

**Effect of Phen on Cd Bioaccumulation in Sediment Exposures**

A randomized experiment including a 4 x 4 factorial treatment arrangement was used to test Phen effects on Cd bioaccumulation in sediments. Ten randomly-selected worms were exposed in 4 replicate 50 mL glass centrifuge tubes containing 15 mL of treatment sediment and
25 mL of overlying APW. Treatment sediments contained 884 mg kg\(^{-1}\) Cd including 45 µCi \(^{109}\)Cd combined with 0, 88, 214 and 414 mg kg\(^{-1}\) Phen (values reflect measured concentrations). Animals were collected from exposure chambers after 6, 12, 24 and 72 h exposure periods, enumerated and rinsed three times with APW. Animals from each replicate were allowed to depurate gut contents for 6 h in individual 100 mL plastic beakers containing 80 mL of APW. After gut clearance, surviving worms from each replicate were rinsed, transferred to clean vials and gamma counted. Animal dry weights were determined for each replicate after radioactivity assays. Counts per minute (cpm) data were corrected for radioactive decay and for gamma-counting efficiency to calculate disintegrations per minute (dpm). The ratio of the measured sediment-Cd concentration to dpm mg\(^{-1}\) was used to determine the dry-weight concentration of Cd found in \(I.\ templetoni\) tissue. Values of \(B_{\text{max}}\) (representing equilibrium tissue-Cd concentrations) including 95% C.I. were calculated for each treatment using a Michaelis-Menten one-site saturation model (SigmaPlot® 8.02, SPSS Inc. Chicago, IL, USA). Non-overlapping 95% C.I. were used as criteria to detect differences between treatments.

Two additional replicates were included as part of the exposure regime described above and were used to test effects of Phen on Cd concentration and pH in overlying water and porewater. At the 6, 12, 24 and 72 h exposure periods, one 2 mL sample of overlying water was removed from each replicate and assayed for \(^{109}\)Cd activity. A second sample of overlying water was filtered through a 0.45 µm membrane filter (Pall Life Sciences) to remove non-dissolved material and assayed for \(^{109}\)Cd activity. The pH of overlying water was measured prior to sampling. Remaining overlying water was decanted and each 50 mL centrifuge tube replicate was centrifuged at 1000 rpm for 10 min to extract porewater. Porewater was removed from each tube using a plastic transfer pipette and a 2 mL sample was assayed for \(^{109}\)Cd activity.
remaining porewater was used to measure porewater pH, filtered as described above and a 2 mL sample was assayed for radioactivity. Radioactivity per unit volume of water was used to determine aqueous-Cd concentrations using methods described above. Unfiltered overlying water and porewater samples represent the bulk Cd concentration in the aqueous medium and filtered samples represent Cd found in the dissolved phase. Effects of Phen and exposure period on pH and Cd concentrations in unfiltered and filtered overlying water and porewater were tested using 2-way ANOVA.

**Cd Effect on Phen Bioaccumulation and BSAF**

Phenanthrene bioaccumulation rates were measured in sediment during the Cd bioaccumulation bioassay described above. Radiolabeled Phen (14C, 15 µCi) was supplemented during standard Phen sediment-loading procedures. An additional block of treatments was run in parallel to the sediment-Cd bioaccumulation experiment described above where worms were exposed to 0 mg kg\(^{-1}\) Cd and 88, 214 and 414 mg kg\(^{-1}\) Phen (measured concentrations) for 12 h. This block of treatments was used to test the effect of Cd on Phen bioaccumulation in worms exposed to Phen alone and the Cd-Phen mixture. The effects of Phen concentration, exposure duration and Cd concentration on biota-sediment accumulation factor (BSAF) were also tested.

\[
\text{BSAF} = \frac{W_{\text{org}} / f_{\text{lipid}}}{W_{\text{sed}} / f_{\text{OC}}} 
\]

(1)

In the BSAF model, \(W_{\text{org}}\) is the concentration of Phen in tissues (µg g\(^{-1}\) dry weight), \(f_{\text{lipid}}\) represents lipid content of worm (g g\(^{-1}\) dry weight), \(W_{\text{sed}}\) is the Phen concentration in sediment (µg g\(^{-1}\) dry weight) and \(f_{\text{OC}}\) represents the organic carbon content (OC) of sediment (g g\(^{-1}\) dry weight). Procedures for determining \(f_{\text{lipid}}\) were determined using a microgravimetric technique.
following Lu et al. (2003). Procedures for determining BSAF were conducted following Millward et al. (2001). Effects of Phen concentration and exposure duration as well as the effects of Cd and Phen concentration on both Phen bioaccumulation and BSAF were tested using 2-way ANOVA (data were natural-log transformed to meet assumptions of ANOVA). Calculations to determine $B_{\text{max}}$ and 95% C.I. values for tissue-Phen concentrations in tissue were calculated using the one-site saturation model described above.

**Effect of Phen on Cd Elimination in Sediment Exposures**

*Ilyodrilus templetoni* was exposed to 812 mg kg$^{-1}$ non-radiolabeled Cd and 0 and 438 mg kg$^{-1}$ Phen in a randomized design experiment. Groups of 20 randomly selected worms were exposed to 15 mL of treatment sediment in 50 mL glass centrifuge vials for 1, 2, 4 and 8 d in 4 replicates. Three additional sets of replicates were included for each sediment treatment in which worms were exposed for 8 d. These worms were used to investigate the effect of exposure to Cd-Phen mixtures on subsequent Cd elimination rates in clean sediments. After exposure to treatment sediments, worms were recovered and surviving worms were enumerated. Worms were allowed to clear gut contents in clean APW for 6 h as described above. After gut clearance, worms used to investigate Cd elimination rates were transferred to 50 mL glass centrifuge tubes containing 15 mL of clean sediment and 25 mL of overlying APW. Worms were recovered from clean sediments after 2, 4 and 8 d, enumerated for survivorship, and allowed to clear gut contents for 6 h. Surviving worms from each replicate were transferred to 13 mL glass vessels, frozen and then freeze dried. After tissue dry weights had been measured, worm tissues were digested and refluxed for 3h in 2 ml of concentrated trace-metal grade HNO$_3$. Ultra-clean Milli-Q$^\circledR$ water was used to bring the total volume of solution in each tissue extraction vessel up to 10 mL. Tissue-Cd concentrations were determined using ICP-AES. The effects of Phen and time on Cd
bioaccumulation were tested using 2-way ANOVA and tissue-Cd $B_{\text{max}}$ values including 95% confidence intervals were calculated the one-site saturation model mentioned above. Elimination rate constants for Cd accumulated from food ($k_{\text{ef}}$) were calculated for both Cd alone and the Cd-Phen mixture as described above.

**Modeling Phen Effects on Cd Bioaccumulation**

A bioenergetic kinetic model (Wang and Fisher, 1999b) was used to examine effects of Phen on the relative contribution of feeding and aqueous uptake to bioaccumulation kinetics in *I. templetoni*.

$$\frac{dC_A}{dt} = [(ku * C_w) + (AE * IR * C_f)] - (k_e + g) * C_A$$

(2)

In the model, $C_A$ represents the concentration of metal in animal tissues ($\mu g \, g^{-1}$), $t$ is the time of exposure, $ku$ represents the uptake rate constant from the dissolved phase ($L \, g^{-1} \, d^{-1}$), $C_w$ is the concentration of metal in the dissolved phase ($\mu g \, L^{-1}$), $AE$ represents assimilation efficiency from ingested material (% assimilation), $IR$ is the ingestion rate ($mg \, g^{-1}$), $C_f$ is the concentration of metal in the ingested material ($\mu g \, mg^{-1}$), $k_e$ is the efflux rate constant ($d^{-1}$), and $g$ is the growth rate constant ($d^{-1}$). Equation 1 was integrated to the following form:

$C_A = A \{1 - \exp[- (k_e + g) * t]\}$

(3)

$$A = \frac{[(ku * C_w) + (AE * IR * C_f)]}{(k_e + g)}$$

(4)

The parameters $ku$, $C_w$, $AE$, $C_f$, and $k_e$ were determined in the bioassays described above while $IR$ and $g$ values were calculated based on the results of Chapter 5.
RESULTS

Effect of Phen on Cd SEM - AVS and Mortality

There were no significant effects of Phen, Cd or their interaction on sediment AVS concentration (P ≥ 0.093 for all tests, Table 6.1). Increases in sediment-Cd concentration significantly increased Cd SEM (P < 0.001). Phen had no effect on Cd SEM (P = 0.678). SEM - AVS relationships for Cd accurately predicted the absence of lethality in *I. templetoni* exposed to Cd and Cd-Phen mixtures. Mortality was low (≤ 6%) in treatments having negative SEM - AVS values, regardless of the presence of Phen (Table 6.1).

Effects of Phen on Aqueous Cd Concentrations

High variability in data within and among treatments coupled with low numbers of replicates reduced the power of statistical comparisons of Cd concentration. However trends were discerned. First, although the relationship between Phen and aqueous Cd concentration appeared random, the presence of Phen did appear to be associated with a relatively high frequency of events where aqueous Cd concentrations were higher than in Cd alone treatments (Figure 6.1). Second, although the concentration of Cd in porewater was substantially higher than was observed in overlying water (Figure 6.1A and 6.1C), a much lower proportion of Cd in porewater was present in the dissolved phase. A comparison of dissolved Cd concentrations in overlying water and porewater indicated the concentrations were relatively similar (Figure 6.1B and 6.1D) among Cd alone and Cd-Phen mixture treatments. Third, the relationship between aqueous Cd concentrations and time appears to be mostly random. Finally, pH in all treatments occupied a narrow range of values (6.39 to 6.66) with no apparent trend between the presence of Phen and pH or time and pH (not displayed).
Figure 6.1. Effect of phenanthrene (Phen) on Cd concentrations in overlying water and in porewater. Bioassay sediments contained 887 mg kg\(^{-1}\) Cd combined with 0, 88, 214 and 414 mg kg\(^{-1}\) Phen. Panels A and C represent bulk-Cd concentrations in overlying water and porewater respectively. Panels B and D represent dissolved-Cd concentrations in overlying water and porewater respectively. All aqueous Cd concentrations are given in \(\mu \text{g L}^{-1}\).
Effect of Phen on Cd Bioaccumulation and Elimination in Water-Only Exposures

Bioaccumulation of Cd in water-only exposures increased linearly with time regardless of Cd concentration or the presence of Phen (Figure 6.2A & 6.2B). Uptake rate constants (k_u) for the 130 µg L\(^{-1}\) Cd exposure were 0.165, 0.179 and 0.174 when combined with the 0, 166 and 431 µg L\(^{-1}\) Phen treatments respectively. In the 271 µg L\(^{-1}\) exposure, k_u values were slightly lower: 0.111, 0.117 and 0.094 when combined with the 0, 166 and 431 µg L\(^{-1}\) Phen treatments respectively. Cadmium concentration, Phen concentration and time had significant effects (P < 0.05) on Cd bioaccumulation in aqueous exposures. There were no significant interactions detected between treatments (P < 0.05). Dunnett’s test indicated that only the highest Phen concentration (431 µg L\(^{-1}\)) caused a significant change in Cd bioaccumulation compared to treatments containing Cd alone. Although the effect of this highest Phen concentration on Cd bioaccumulation was indicated to be statistically significant, the k_u values within Cd treatments were very similar.

Exposure to Cd-Phen mixtures in water-only exposures had no appreciable effect on Cd elimination rates compared to worms exposed to Cd alone. Tissue-Cd concentrations decreased linearly with time and at a slow rate (2.1% to 4.8% loss d\(^{-1}\)). Elimination rate constants (k_{ew}) for the 130 µg L\(^{-1}\) Cd exposure were 0.023, 0.021 and 0.041 for the 0, 166 and 431 µg L\(^{-1}\) Phen treatments respectively. In the 271 µg L\(^{-1}\) exposure, k_{ew} values included: 0.025, 0.048 and 0.031 for the 0, 166 and 431 µg L\(^{-1}\) Phen treatments respectively.

Effects of Phen on Cd Elimination in Sediment

Cadmium bioaccumulation rates were significantly higher (P < 0.001) in Cd alone treatments than in treatments containing Cd combined with 438 mg kg\(^{-1}\) Phen (Figure 6.3A). treatments than when combined with Phen (P < 0.001). The B_{max} value for tissue-Cd
Figure 6.2. Effect of phenanthrene (Phen) on Cd bioaccumulation and elimination in *Ilyodrilus templetoni* in water-only exposures. *I. templetoni* was exposed to 130 and 271 µg L⁻¹ Cd combined with 0, 166 and 432 µg L⁻¹ Phen. Panels A and C represent Cd bioaccumulation kinetics and panels B and D represent Cd elimination kinetics after 12 h exposure. Cd elimination occurred in clean water.
Figure 6.3. Effect of phenanthrene (Phen) on Cd bioaccumulation and elimination in *Ilyodrilus templetoni* in sediment exposures. Cd bioaccumulation was measured in *I. templetoni* exposed to 812 mg kg\(^{-1}\) Cd combined with 0, and 438 mg kg\(^{-1}\) Phen (Panel A and B). After 8 d exposure, worms were allowed to eliminate Cd in clean sediment for 1, 2, 4, and 8 d intervals (Panel B).
concentration was 99.7 µg g⁻¹ (73.2-126.2, 95% C.I.) for worms exposed to Cd alone and 30.1 µg g⁻¹ (18.2-42.7, 95% C.I.) for the Cd-Phen mixture. Cadmium body burdens in worms were not significantly reduced over the 8 d elimination period and unaffected by the presence of Phen (P > 0.05, Figure 6.3B). Cadmium elimination rate constants (kₑᶠ) for *I. templetoni* exposed to contaminated sediment (food) and allowed to eliminate Cd body burdens in clean sediment were 0.031 and 0.030 for Cd alone and Cd-Phen mixtures respectively.

**Effect of Phen on Cd Assimilation Efficiency**

The presence of Phen significantly reduced (P < 0.001) Cd AE from sediments in *I. templetoni* (Figure 6.4B). In worms that had not been pre-exposed to Cd, Phen reduced Cd AE from 52% to 10% and in pre-exposed worms from 31% to 9%. Pre-exposure to Cd had no affect on Cd AE (P = 0.185), and there was no detectible interaction between treatments (P = 0.290).

**Modeling Phen Effects on Cd Bioaccumulation**

Results of bioenergetic kinetic modeling suggested increases in sediment-Phen concentrations in combination with Cd reduce Cd bioaccumulation in *I. templetoni* (Figure 6.5). A comparison of predicted tissue-Cd concentrations at d-4 suggested that worms exposed to Cd alone would be expected to have greater than two orders of magnitude higher tissue-Cd concentrations than animals exposed to the mixture of Cd and “high” Phen. Contributions to Cd bioaccumulation from the dissolved phase are suggested to be minimal (5% or less). The model also suggested that equilibrium concentrations of Cd in *I. templetoni* tissues would not be approached until well after 100 d of exposure. A summary of model parameter values is given in Table 6.2.

**Test of the Kinetic Model**

In sediment exposures Phen increased the bioaccumulation of Cd into *I. templetoni* tissues (Figure 6.6). Both Phen and time had significant effects on Cd bioaccumulation
Figure 6.4. Effect of phenanthrene (Phen) on Cd assimilation efficiency (AE) from sediments in *Ilyodrilus templetoni*. A pulse-chase method was used to calculate AE (See Materials and Methods for description). Panel A represents the depuration kinetics of *I. templetoni* after 40 min exposure to Cd. Panel B represents AE values as percentages.
Figure 6.5. Kinetic model predictions for phenanthrene (Phen) affects on Cd bioaccumulation in *Ilyodrilus templetoni*. See Materials and Methods for model description and table 6.2 for empirically derived model parameters.

(P <0.05) and there were no significant interactive effects between Phen and time. The $B_{max}$ values (including 95% C.I. in parentheses) for Cd in tissues were 132 (0-431), 112 (89-136), 165 (112-217) and 211 (154-269) µg g$^{-1}$ when exposed to sediments containing 887 mg kg$^{-1}$ Cd including 0, 88, 214 and 414 mg kg$^{-1}$ Phen respectively. Bioaccumulation kinetics suggested that tissue-Cd equilibria were approached rapidly (~24 h) in treatments including Phen. Bioaccumulation kinetics suggested that tissue-Cd equilibrium was not approached during the 96 h bioassay in the Cd alone treatment (Figure 6.6).
Table 6.2. Model parameter values used for investigation of Phen effects on Cd bioaccumulation kinetics. Definitions and derivations of model parameters and bioenergetic kinetic model can be found in the Materials and Methods section. Values for IR were determined from results of Gust and Fleeger (submitted).

<table>
<thead>
<tr>
<th>Phenanthrene conc.</th>
<th>K_u</th>
<th>C_w</th>
<th>AE %</th>
<th>IR</th>
<th>C_f</th>
<th>K_eW</th>
<th>K_ef</th>
<th>g</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.16</td>
<td>12.8</td>
<td>30.5</td>
<td>9062</td>
<td>0.878</td>
<td>0.023</td>
<td>0.031</td>
<td>0.000</td>
</tr>
<tr>
<td>Low</td>
<td>0.18a</td>
<td>33.0</td>
<td>8.7</td>
<td>8410</td>
<td>0.878</td>
<td>0.021a</td>
<td>0.030c</td>
<td>-0.003</td>
</tr>
<tr>
<td>Medium</td>
<td>0.18</td>
<td>40.2</td>
<td>8.7b</td>
<td>5605</td>
<td>0.878</td>
<td>0.021</td>
<td>0.030c</td>
<td>-0.014</td>
</tr>
<tr>
<td>High</td>
<td>0.17</td>
<td>40.3</td>
<td>8.7b</td>
<td>2365</td>
<td>0.878</td>
<td>0.041</td>
<td>0.030</td>
<td>-0.027</td>
</tr>
</tbody>
</table>

a No empirical value available. The parameter value for "Medium Phen" was used for kinetic model calculations.

b No empirical value available. The parameter value for "Low Phen" was used for kinetic model calculations.
c No empirical value available. The parameter value for "High Phen" was used for kinetic model calculations.

Cd Effect on Phen Bioaccumulation and BSAF

Cadmium had no effect (P = 0.198) on Phen bioaccumulation regardless of Phen concentration (Figure 6.7B). Increases in sediment-Phen concentrations resulted in significant increases (P < 0.001) in Phen bioaccumulation (Figure 6.7A). There was no significant effect (P = 0.084) of exposure duration on Phen bioaccumulation suggesting that worms had achieved equilibrium Phen-tissue concentrations within 6 h. The B_{max} values (including 95% C.I. in parentheses) for treatments were 154 (127-182), 272 (205-340) and 586 (415-758) for worms exposed to 88, 214 and 414 mg kg^{-1} Phen respectively. The effect of exposure duration and Phen concentration on BSAF were variable within and between treatments (Figure 6.7C). Results of ANOVA suggest Phen concentration and duration of exposure did not significantly
Figure 6.6. Effect of phenanthrene (Phen) on Cd bioaccumulation in *Ilyodrilus templetoni* in sediment exposures. Cd bioaccumulation was measured in *I. templetoni* exposed to 887 mg kg\(^{-1}\) Cd combined with 0, 88, 214 and 414 mg kg\(^{-1}\) Phen.

effect BSAF (P = 0.063 and 0.188 respectively), but that their interaction did (P = 0.027).

Cadmium was not observed to have a significant effect on BSAF (Figure 6.7D) regardless of Phen concentration (No P value below 0.251).

**DISCUSSION**

**Effects of Phen on Cd Bioavailability**

The bioavailability of contaminants in natural environments strongly influences contaminant toxicity in native organisms. It has been demonstrated that no significant mortality
Figure 6.7. Effect of Cd on phenanthrene (Phen) bioaccumulation in *Ilyodrilus templetoni* in sediment exposures. *I. templetoni* were exposed to 887 mg kg\(^{-1}\) Cd in combination with 0, 88, 214 and 414 mg kg\(^{-1}\) Phen. Panels A and C represent Phen bioaccumulation kinetics and biota sediment accumulation factor (BSAF) kinetics. Panels B and D compare Phen bioaccumulation and BSAF in organisms exposed to Phen alone and the Cd-Phen mixture after 12 h exposure.
is expected in benthic invertebrates exposed to sediments when the molar concentration of AVS is higher than the molar concentration of simultaneously extracted Cd (Di Toro et al. 1992). In the present study, Phen was not observed to alter AVS or simultaneously extractable Cd concentrations in sediment bioassays (Table 6.1). As was observed in Chapter 5, lethality in sediment exposures was highest in *I. templetoni* exposed to Cd alone compared to treatments containing Cd-Phen mixtures. As is suggested by the model, only background levels of mortality were observed in treatments were AVS concentrations were higher than the concentration of simultaneously extractable Cd regardless of the presence of Phen. The model is less accurate at predicting when lethality will occur, so it is not as useful for investigation of antagonistic effects between contaminants. Phenanthrene had no effect on pH, AVS or the SEM of Cd, therefore Phen would not be expected to alter the bioavailability and/or toxicity of Cd.

Results from analyses of Cd porewater and overlying water suggested Phen effects on Cd concentrations were neither consistent nor strong (Figure 6.1). Bulk concentrations of Cd measured in the aqueous phase of porewater were approximately 2-fold higher than bulk-Cd concentrations detected in overlying water (Figure 6.1A and 6.1C). When overlying water and porewater were filtered to remove non-dissolved materials, Cd concentrations were observed to be roughly equivalent (Figure 6.1B and 6.1D). It has been suggested that metals bound to various water-borne ligands are less bioavailable than ionized metal, and that ionized metal is ultimately the most bioavailable metal fraction (Di Toro et al 2001, Chapman et al 1998). In the present study, treatments containing Cd alone tended to have less Cd dissolved in porewater and overlying than was measured in Cd-Phen mixture treatments. These results and the results of SEM - AVS relationships indicate that Cd-bioavailability is not affected by Phen in a way that would be expected to elicit antagonistic toxicity.
Cd Effect on Phen Bioaccumulation and BSAF

Cadmium had no effect on Phen bioaccumulation or the BSAF for Phen in sediment exposures (Figure 6.7B and 6.7D). Similarly, Moreau et al. 1999 observed that Zn had no effect on Phen bioaccumulation in sheepshead minnow (*Cyprinodon variegatus*). Increasing concentrations of Phen in sediments resulted in increased Phen bioaccumulation, and had variable effects on the BSAF for Phen. Millward et al. (2001) demonstrated that the BSAF for pyrene decreased with increasing sediment-pyrene concentrations in the freshwater oligochaete *Limnodrilus hoffmeisteri*. Although this trend was not observed in the present study, BSAF values for the lowest concentration of Phen in sediment (88 mg kg⁻¹) did tend to increase over time whereas BSAF remained constant or decrease with time in worms exposed to 414 and 214 mg kg⁻¹ respectively. Increases in pyrene BSAF over time were also observed for low concentrations of pyrene in *L. hoffmeisteri* (Millward et al. 2001). Cadmium has been shown to interfere with cytochrome P450 mediated PAH metabolism in a variety of fish species (van den Hurk 1999a, Viarengo et al 1997, Förlin et al 1986). Cytochrome P450 mediated metabolism of PAHs can induce production of bioactivated metabolites that have greater toxicity than the parent compound (Casarett and Doull, 2001). Although Phen bioaccumulation was unaffected by Cd in the current study, Cd-mediated reductions in toxic-intermediate production may confer reduced toxicity. This relationship remains to be tested in annelids.

Effect of Phen on Cd Bioaccumulation in Water-Only Exposures

Phenanthrene had only minor effects on Cd bioaccumulation rates in water-only exposures (Figure 6.2). Worms exposed to solutions of Cd at 271 µg L⁻¹ bioaccumulated more Cd than worms exposed to 130 µg L⁻¹ Cd regardless of the presence of Phen. Although Cd bioaccumulation was highest when exposed to the highest water-Cd concentration, the bioaccumulation rate constant (κₐ) for Cd was greater in treatments containing the lowest water-
Cd concentration (130 µg L\(^{-1}\)). These results suggest that Cd bioaccumulation from the dissolved phase may be rate limiting in \(I.\) templetoni. Although Cd uptake from the dissolved phase contributes to Cd bioaccumulation in \(I.\) templetoni, several studies have suggested that the contribution of Cd uptake from the dissolved phase to total Cd bioaccumulation is minor (1-5%) in deposit-feeding annelids (Wang and Fisher 1999, Selck et al 1998, Warren et al 1998). Dietary uptake has been shown to be the predominant source of Cd bioaccumulation in these organisms. Therefore, it is likely that the minor effect of Phen on Cd bioaccumulation from the dissolved phase played a negligible role in the antagonistic toxicity observed in \(I.\) templetoni.

**Effects of Phen on Cd Elimination in Sediment**

Cadmium elimination rate constants \((k_{ew})\) for \(I.\) templetoni exposed in water-only exposures were low \((\leq 4.1\% \text{ d}^{-1})\) and not appreciably affected by the presence of Phen. Likewise, Cd elimination after exposure to contaminated sediments indicated Phen had negligible effects on Cd elimination rate constants \((k_{es})\) and elimination rates were also slow \((\leq 3.1\% \text{ d}^{-1})\). Cadmium elimination kinetics of similar magnitude have also been observed in the marine polychaete \(Capitella\) species I (Selck et al. 1998). Elimination is not considered to be a major mechanism by which annelids mitigate the toxic effects of Cd body burdens. Although annelids have not been observed to eliminate Cd efficiently, they are able to detoxify bioaccumulated Cd by sequestering the metal into metallothionein complexes and into metal rich granules (Klerks and Bartholomew 1991, Wallace et al. 1998). Because Phen had little effect on Cd elimination rate in both water-only and in sediments, it is unlikely that Phen appreciably effected elimination of bioaccumulated Cd. Therefore, Phen induced modifications in Cd bioaccumulation and effects of the contaminant mixture on lethality are not explained by Cd elimination rate.
**Effect of Phen on Cd Assimilation Efficiency**

Assimilation efficiency (AE) of Cd was reduced in Cd-Phen mixtures compared to worms exposed to Cd alone (Figure 6.4). The AE of Cd in *I. templetoni* exposed to Cd alone was slightly higher than was found in the deposit feeding marine polychaete *Nereis succinea* (Wang et al. 1999). *Ilyodrilus templetoni* has been observed to ingest sediment at a rate multiple times its body weight d⁻¹ (Chapter 5). Because ingestion rates in bulk-deposit feeding invertebrates, such as *I. templetoni*, are generally high, it was hypothesized that AE may be reduced during prolonged exposure to Cd as equilibrium tissue-Cd concentrations were approached. Pre-exposure to Cd did not significantly reduce Cd assimilation efficiency, regardless of the presence of Phen. Even at the lowest observed AE determined for Cd (9%), the high feeding rate of *I. templetoni* could easily result in a high proportion of Cd bioaccumulation resulting from uptake via ingestion (>90%). The sustained reduction in Cd AE observed in Cd-Phen mixtures compared to worms exposed to Cd alone would likely cause a major reduction in Cd bioaccumulation.

**Test of Kinetic Model**

The Cd bioaccumulation kinetics experiment used to test of the kinetic model indicated Cd bioaccumulation rates increased with increasing Phen concentrations (Figure 6.6A). Although this trend was observed through the 96 h exposure, B_max values indicated Cd body burdens may not be different once worm tissues reach equilibrium with the environment. Conversely, Cd bioaccumulation kinetics observed in the elimination rate experiment indicated Phen reduced Cd bioaccumulation rates (Figure 6.3A). In that experiment, Cd body burdens were nearly 4x lower in animals exposed to Cd-Phen mixtures than in animals exposed to Cd alone and equilibrium was approached quickly (within 1 d) regardless of the presence of Phen.
**Evaluation of Kinetic Model Predictions:**

A comparison of model predictions (Figure 6.5) and tissue-Cd concentrations measured in *I. templetoni* exposed in sediment bioassays (Figure 6.3A and 6.6) indicated the model overestimated Cd bioaccumulation rates by as much as 2 orders of magnitude. The kinetic model suggested equilibrium Cd body burdens would not be approached until after over 100 d of exposure. Although some treatments in the test of the kinetic model did appear to require a long time period to reach equilibrium (Figure 6.6), most treatments did appear to reach equilibrium within a relatively short amount of time (1-8 d).

A combination of factors were responsible for the inaccuracies in model predictions described above. In order to better understand why these predictions were inaccurate, and as well to elaborate on why certain trends suggested by the kinetic model appear correct, we must consider *I. templetoni*’s interaction with sediments and how these interactions may influence model predictions. First, the bioenergetic model consists of 3 major compartments that effect metal body burden: (1) metal uptake from sediment/food, (2) metal uptake from the dissolved phase, and (3) elimination of metal from tissues (Wang and Fisher, 1999). *Ilyodrilus templetoni* is a bulk-deposit feeder that may ingest sediment at rates in excess of 9x its body weight d⁻¹ (Chapter 5). This feeding strategy puts *I. templetoni* in contact with high levels of contaminant inside of the gut. Gut fluids of annelids have been observed to extract metals from sediment efficiently (Chen et al 2000). Extraction of metals from sediment may increase metal absorption and therefore metal assimilation into tissues. The combination of bulk-deposit feeders’ extremely high ingestion rates combined with even a modest AE can result in greater than 95% of metal bioaccumulation from ingested sediment (Wang and Fisher 1999). The model suggested this trend was also true for Cd bioaccumulation in *I. templetoni*. Contributions to Cd
bioaccumulation from the dissolved phase were minor considering the low Cd concentrations measured in overlying water and porewater (Figure 6.1) coupled with the modest uptake rate constant (\(k_u\)) observed in the dissolved phase (Table 6.2, Figure 6.2).

Assimilation efficiency (AE) of Cd in *I. templetoni* ranged from approximately 8% in Cd-Phen mixtures to as high as 50% for Cd alone. The observation that pre-exposure to Cd had no effect on AE regardless of the presence of Phen suggests that AE likely remains constant over time. Feeding rate (IR) in *I. templetoni* (as well as in other annelids) has been observed to decrease with increasing Phen concentrations in sediment (Chapter 5, Lotufo and Fleeger 1996). Phen-mediated reductions in both IR and AE were greatly responsible for the model prediction of reduced Cd bioaccumulation with increasing sediment-Phen concentrations (Figure 6.5). Although the test of the bioenergetic kinetic model provided unexpected results, Cd bioaccumulation kinetics observed in the sediment Cd elimination experiment indicated the model did predict patterns of Phen affects on Cd bioaccumulation (Figure 6.3A).

When incorporated into the kinetic model, slow elimination rates increase the time necessary for contaminants to reach equilibrium in tissues. Additionally, elimination rates affect the magnitude of contaminant expected to be bioaccumulated in tissues at equilibrium. Cadmium elimination rates in *I. templetoni* were very slow and unaffected by the presence of Phen (Figures 6.2B, 6.2D and 6.3B). In concert with the heavy bioaccumulation of Cd from feeding, the slow elimination rate of Cd from tissues resulted in both the high predicted equilibrium tissue-Cd concentrations and the slow approach to tissue equilibrium.

To summarize the relationship between the kinetic model and the empirically derived bioaccumulation data, we must consider the contribution of each model parameter to predicted bioaccumulation and the inter-experiment variability in *I. templetoni*‘s interactions with contaminated sediments. First, model predictions indicate that > 90% of Cd bioaccumulation
occurs via uptake from feeding whereas Cd bioaccumulation from the dissolved porewater phase contributes < 10%. In our experiments, the parameters associated with Cd bioaccumulation from the dissolved phase (k_u and C_w) were marginally affected by the presence of Phen and contribute relatively little (< 10%) to overall Cd bioaccumulation. Hence, Phen did not affect predicted total Cd bioaccumulation via affecting uptake from the dissolved phase. As mentioned above, Phen affects on Cd elimination (k_e and k_f) were minimal and therefore, Phen did not appreciably alter predicted Cd bioaccumulation kinetics. Phenanthrene greatly reduced ingestion rate (IR), thereby reducing exposure to sediment-associated Cd. Although reduced IR may serve to decrease predicted Cd bioaccumulation from the feeding route of uptake, decreased IR likely increased sediment residence time in the gut which is generally associated with increases in assimilation efficiency (AE). However, our results suggest that Phen actually reduced Cd AE. Given that > 90% of Cd bioaccumulation is predicted to arrive via feeding and that Phen had no effect on the concentration of Cd in sediment (food, C_f), Phen decreased IR by as much as 74% and Phen reduced Cd AE by 71%, the Phen-mediated reduction in predicted Cd bioaccumulation is the result of decreased Cd bioaccumulation from feeding.

The effect of Cd-Phen mixtures on worms’ feeding rates (IR) in 10 d experiments was highly consistent among experiments (Chapter 5). As well, elimination rates (k_e and k_f) were consistently low and weakly affected by Phen. Uptake rate constants (k_u) derived from water-only experiments are consistent with the literature and are marginally affected by Phen. Finally, sediment-Cd concentrations (C_f) were unaffected by Phen and dissolved Cd concentrations in overlying water and porewater (C_w) were weakly affected by Phen and are not predicted to contribute strongly to total Cd bioaccumulation. However, effects of Phen on AE and IR in short-term experiments (hourly feeding rate) have been difficult to quantify and have yielded a
high degree of variability among experiments. For example, 3 separate bioassays were conducted in attempt to quantify Phen effects on Cd AE in \textit{I. templetoni} (the third of which is presented in the this study). In the first experiment, \textit{I. templetoni} depurated very little Cd after 40 min exposure resulting in AE values as high as 90%. Alternatively, in the second experiment animals did not feed during the 40 min exposure period resulting in no Cd uptake at all. The variability in results among experiments may have resulted from varying sensitivity to handling stress among cultures, variability in the health of laboratory cultures over time or variability in the microenvironment in experimental treatments that has not been quantified. Regardless of the cause, because IR and AE play such a critical role in contributing to total Cd bioaccumulation, even slight differences among experiments may contribute to large variations in results among experiments. This suggests that even when using identical experimental protocols among experiments, the short-term feeding behavior / digestive physiology of \textit{I. templetoni} in sediment exposures may ultimately control Cd bioaccumulation.

**Summary and Conclusions**

In this study, Phen was not observed to affect parameters associated with Cd bioavailability which is consistent with Gust (in press). Also, Cd was not observed to affect Phen bioaccumulation kinetics and BSAF in \textit{I. templetoni}. The antagonistic toxicity observed between Cd and Phen Chapter 5 is more likely related to alterations in exposure and bioaccumulation of Cd than a true pharmacological interaction. The presence of Phen caused heavy reductions in sediment feeding and greatly reduced the Cd assimilation efficiency in sediments. These effects were associated with both predicted and observed reductions in Cd bioaccumulation rates. Metal bioaccumulation rates directly influence lethality caused by metal contaminants (Barron et al 2002). Based on these results, it can be concluded that apparent
interactive effects of chemical mixtures in sediment environments may occur via mechanisms related to changes in exposure and contaminant bioaccumulation.

**LITERATURE CITED**


Gust KA (in press) Joint toxicity of cadmium and phenanthrene in the freshwater amphipod *Hyalella azteca*. Arch Environ Contam Toxicol


Moreau CJ, Klerks PL, Haas CN (1999) Interaction between phenanthrene and zinc in their toxicity to the sheepshead minnow (*Cyprinodon variegatus*). Arch Environ Contam Toxicol 37:251-257


Viarengo A, Bettella E, Fabbri R, Burlando B, Lafaurie M (1997) Heavy metal inhibition of EROD activation in liver microsomes from the bass *Dicentrarchus labrax* exposed to organic xenobiotics: Role of GSH in the reduction of heavy metal effects. Mar Environ Research 44:1-11


CHAPTER 7

SUMMARY AND CONCLUSIONS
The comprehensive literature review presented in Chapter 2 provides a weight-of-evidence view that the hypothesis of independent joint toxicity for metal-PAH mixtures is inaccurate. Over 70% of the papers cited suggest that the joint toxicity of metal-PAH mixtures may be interactive. Examples of interactive effects range from interference with enzymatic pathways that detoxify PAH by metals, and vice versa, through ecosystem level effects (Chapter 2). Within the literature-base (which consists of just over 30 papers), the connectivity between true toxicological interactions and observed interactive effects of metal-PAH mixtures in individuals, populations, communities and ecosystems is difficult to characterize. The results of this dissertation suggest that effects of metal-PAH mixtures in benthic invertebrates are endpoint specific, species specific, and specific to the type of environment in which organisms are exposed. Further, the results suggest that although the toxicology of metal-PAH mixtures may be independent, observed interactive effects are be manifested through mechanisms related to mixture-mediated changes in contaminant exposure and contaminant bioaccumulation.

In Chapter 3, the freshwater amphipod *Hyalella azteca* was exposed to sediment-amended Cd and phenanthrene (Phen) individually and in combination using US Environmental Protection Agency (USEPA) 10-d sediment toxicity bioassays with lethality and growth endpoints. The lethal joint toxicity of Cd and Phen was investigated separately in 24, 48 and 72-h aqueous exposures. In sediment exposures, a sublethal concentration of Phen (144 mg kg\(^{-1}\)) in combination with Cd increased mortality over a range of Cd concentrations reducing the 10-d LC\(_{50}\) for Cd from 523 mg kg\(^{-1}\) (461-588, 95% C.I.) to 263 mg kg\(^{-1}\) (214-312, 95% C.I.). In contrast, sublethal concentrations of Phen had no effect on the lethal toxicity of Cd in aqueous exposures. Combined sediment-amended Cd and Phen acted independently on growth rate. Rate reductions were driven primarily by Cd. Our findings indicate that association with
sediment influences the joint toxicity of Cd and Phen. Thus, Cd and Phen mixtures may cause synergistic or independent toxicity in *H. azteca* depending on the endpoint investigated and the experimental protocol employed. As an implication of these results, the interpretation of standardized toxicity bioassays, including whole effluent toxicity (WET) tests and single compound toxicity tests, must be made with caution. These assessment protocols may underestimate potentially hazardous mixture effects in sediment environments. Therefore, risk assessment protocols for environments containing metal-PAH mixtures must include robust methods that are able to detect possible interactive effects among contaminants to optimize environmental protection.

The purpose of Chapter 4 was to identify the causes of the synergistic effects observed in Chapter 3. Standard USEPA procedures were applied and extended to test effects of Phen on sediment-Cd uptake, aqueous-Cd uptake and Cd-elimination kinetics in the amphipod *Hyalella azteca*. Parameters indicative of Cd bioavailability were also measured. Results indicated: (1) In sediment exposures, Phen increased the projected equilibrium-tissue concentration of Cd from 47.2 (36.2-58.3, 95% C.I.) to 221.1 µg g⁻¹ (117.8-324.3, 95% C.I.). (2) Dissolved Cd concentrations in overlying water were low (2.6-5.3 µg L⁻¹) in sediment bioassays and were unaffected by Phen. (3) Phenanthrene had no effect on Cd bioaccumulation or Cd-elimination kinetics in water-only exposures. These results suggest enhanced Cd bioaccumulation in Cd-Phen mixtures occurred via a sediment-mediated process, and likely resulted from increased uptake associated with feeding (by Phen-induced alterations in ingestion or digestive processes). Observed increases in *H. azteca* lethality when exposed to Cd-Phen mixtures in sediment, but not in water-only exposures, likely resulted from increased Cd bioaccumulation rate rather than a true toxicological synergism. Thus, apparent synergisms or antagonisms in sediment may be a function of exposure-mediated effects that are unrelated to toxicological interactions.
In Chapter 5, Sediment bioassays were utilized to quantify effects of individual and combined contaminants in the bulk-deposit feeding oligochaete *Ilyodrilus templetoni*. The 10-d LC$_{50}$ for Cd alone was 1375 mg kg$^{-1}$ (95% C.I. 1340-1412), while Phen elicited no mortality even when loaded to sediment saturation. Combined contaminants elicited antagonistic lethal effects and independent responses for feeding rate (measured as sediment ingestion). The presence of Phen decreased lethality, increasing the LC$_{50}$ of Cd by as much as 40%. Regression analyses indicated Phen was nearly 10 times more potent than Cd in eliciting feeding rate reductions. Exposure to Cd-Phen mixtures resulted in feeding rate reductions equivalent to those caused by Phen alone. The marked reduction in sediment ingestion induced by the co-pollutant Phen reduced exposure to Cd via ingestion. We suggest that this Phen-induced reduction in Cd exposure decreased Cd bioaccumulation and subsequent lethality. More generally, we suggest that even if the toxicological effects among dissimilarly-acting chemicals (including metals and hydrocarbons) are independent, contaminant mixtures may elicit unexpected interactive effects facilitated by modifying exposure.

In Chapter 6, a bioenergetic kinetic model, bioaccumulation kinetics and tests of contaminant bioavailability were utilized to characterize the source of antagonistic toxicity observed between Cd and Phen in *I. templetoni* (Chapter 5). A series of sediment and water-only bioassays were conducted to determine Phen effects on metrics of Cd bioavailability, Cd bioaccumulation kinetics, Cd elimination kinetics and parameters associated with the kinetic model. Effects of Cd on Phen bioavailability were tested using biota sediment accumulation factor (BSAF). Effects of Cd on of Phen bioaccumulation kinetics were tested empirically using radiotracer bioassays. Cadmium did not affect Phen bioaccumulation or BSAF in *I. templetoni*. Phenanthrene had no effect on simultaneously extractable metals - acid volatile sulfide (SEM –
AVS) relationships and had marginal affects on dissolved Cd concentrations in overlying water and porewater. Cadmium assimilation efficiency (AE), sediment-ingestion rate (IR) and growth rate (g) decreased in the presence of Phen. However, Phen had no appreciable effects on all other parameters associated with the kinetic model. Although the kinetic model overestimated Cd bioaccumulation rate and time to tissue-Cd equilibrium, it accurately predicted Phen-mediated reductions in Cd bioaccumulation rates observed in a Cd bioaccumulation experiment. These results suggest that the antagonism observed between Cd and Phen in sediment mixtures likely resulted from decreased Cd bioaccumulation rate. We suggest the antagonism is more likely an exposure-related effect than a true pharmacological interaction. Finally, contaminant-induced changes in feeding behavior and/or digestive physiology appear to be predominant in controlling exposure, bioaccumulation and toxicity.

In summary, the research presented in this dissertation indicates that effects of metal-PAH mixtures are not well characterized by the hypothesis of independence and are species specific, endpoint specific, and specific to the environment type in which organisms are exposed. A comparison of results from chapters 3 and 4 indicates that “biology” influences the joint toxicity combined contaminants. *Hyalella azteca* and *I. templetoni* were each exposed for 10 d to identical contaminants and sediments using nearly identical experimental protocols, but had opposite lethal responses (synergistic in *H. azteca* and antagonistic in *I. templetoni*). Examples of endpoint specificity were observed in both *H. azteca* and *I. templetoni*. For example, although both species experienced interactive effects in sediment exposures, affects of sediment-associated metal-PAH mixtures on feeding rate in *I. templetoni* and growth rate in *H. azteca* were each independent. Observed interactive effects in both *H. azteca* and *I. templetoni* occurred in sediment exposures, but not in water-only exposures indicating that exposure source
influences observed mixture effects. Although standard analytical metrics for assessing metal bioavailability were largely unaffected by the presence of PAH, metal bioaccumulation patterns were greatly altered by PAH in both *H. azteca* and *I. templetoni*. Specifically, Cd bioaccumulation rates in *H. azteca* were greatly increased by the presence of Phen, whereas Phen decreased Cd bioaccumulation in *I. templetoni*. These Phen-mediated alteration in Cd bioaccumulation corresponded with, and were the likely mechanisms responsible for the synergistic and antagonistic lethality observed in *H. azteca* and *I. templetoni* respectively.

In conclusion, the current basis for assessing ecotoxicological effects of contaminant mixtures in natural environments relies heavily on models derived from dosage-based mixture toxicology with considerably less emphasis on environmental science and biology. Understanding how contaminants interact toxicologically is important, but does not provide all the information necessary for assessing effects in natural populations that encounter contaminant mixtures in a diversity of natural environments. My experiments indicate that exposure source may be more important than dosage-based toxicological interactions in determining contaminant mixture effects in sediment environments. If this trend is widespread, understanding how species are exposed, determining the route of uptake and understanding how environmental characteristics affect exposure may be more important in determining mixture effects than mixture toxicology.
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Kurt Gust
Department of Biological Sciences
Louisiana State University
Baton Rouge, LA 70803
Phone: (225) 578-1738
Fax: (225) 578-2597
kgust1@lsu.edu

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VITA

Kurt A. Gust was born September 16, 1976, in Saginaw, Michigan. Kurt graduated from Saginaw Valley State University in 1999 completing a bachelor of science degree in biology with a minor in chemistry. In August of 1999 Kurt was accepted into the doctoral program at Louisiana State University within the Department of Biological Sciences. Presently, Kurt is a candidate for the Doctor of Philosophy degree and will graduate May 20, 2005.